Universidad Nacional Autónoma de México
Facultad de Química

Caracterización del sistema calmodulina-proteinas aceptoras de calmodulina en células germinales masculinas

Tesis que para obtener el grado de
Doctor en Ciencias Químicas (Bioquímica) presenta
M. en C. Raquel Trejo Albarrán

Tutores:
Dr. John R. Dedman
Dr. Antonio Peña Díaz

Ciudad Universitaria
1996

TESIS CON
FALLA DE ORIGEN

UNAM – Dirección General de Bibliotecas Tesis Digitales Restricciones de uso

VNIVERSIDAD NACIONAL
AVTÓNOMA DE
MEXICO

FACULTAD DE QUÍMICA
DIRECCIÓN

LIC. ANTONIO DÍAZ GARCÍA
Jefe de la Unidad de Registro e Información
Ciudad Universitaria
P r e s e n t e .

Me es grato informarle que la alumna, RAQUEL TREJO ALBARRÁN, presentará próximamente su examen para obtener el grado de Doctor en Ciencias Químicas (Bioquímica), ante el siguiente jurado:

Presidente:	Dra. Marieta Tuena Sangri
Primer Vocal:	Dr. Edmundo Chávez Cossio
Segundo Vocal:	Dra. Victoria Chagoya de Hazas
Tercer Vocal:	Dr. Adolfo García Sainz
Secretario:	Dra. Marina Gavilanes Ruiz
Primer Suplente:	Dr. Diego González Halphen
Segundo Suplente:	Dr. Fernando Montiel Aguirre

Sin otro particular de momento, aprovecho la ocasión para enviarle un cordial saludo.

A t e n t a m e n t e
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, D. F. a 12 de abril de 1996.

DR. ANDONI GARRITZ RUIZ
Director.

C.c.p.- Integrantes del Jurado
C.c.p.- Coordinador de Área
C.c.p.- Departamento de Control Escolar
C.c.p.- Interesado
*ggm.

Este trabajo se realizó en el Departamento de Fisiología y Biología Celular de la Escuela de Medicina de la Universidad de Texas en Houston, en el Departamento de Biología Celular del Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional y en la Unidad de Investigación Médica en Enfermedades Oncológicas del Hospital de Oncología, Centro Médico Nacional, Instituto Mexicano del Seguro Social.

Tutor académico : Dr. John R. Dedman.
Tutor oficial: Dr. Antonio Peña Díaz.

Deseo expresar mi reconocimiento y gratitud al Dr. John R. Dedman por sus enseñanzas y por su generoso donativo de reactivos después del sismo de 1985.

También agradezco sinceramente la confianza y el apoyo brindados por el Dr. Antonio Peña Díaz para la realización de este trabajo.

In memoriam

A mi mamá

A mi papá

A Graciela
A Rodolfo

RESUMEN

Se ha observado que el sistema transmisor de señales mitogénicas que utiliza al Ca^{2+} como segundo mensajero y a la calmodulina (CaM) como mediadora de sus efectos, presenta modificaciones en las proteínas blanco de Ca^{2+}-CaM en forma coordinada con el nivel de CaM. En algunos órganos, como cerebro y testículo, se ha observado un aumento en el contenido de CaM durante el desarrollo postnatal, pero no se han identificado los cambios en el sistema Ca^{2+}-CaM-proteínas aceptoras.

Con base en estas observaciones, se estudió el sistema CaM-proteínas aceptoras de Ca^{2+}-CaM en el testículo de la rata en desarrollo. El contenido y distribución subcelular de CaM, así como el de sus proteínas blanco se correlacionó con la velocidad de crecimiento y con la diferenciación alcanzada por el epitelio germinal.

Se observaron tres incrementos en el nivel testicular de CaM: entre los 5-10 días, a los 24 y a los 32 días de edad. El segundo y tercer incrementos se presentaron en la fase exponencial del crecimiento testicular. El primer incremento coincidió con la proliferación de las espermatogonias y de las células de Sertoli. El segundo, con la evolución de los espermatocitos hacia los últimos estadios de la primera profase, la terminación de la división meiótica y la aparición de las espermátidas. El tercero, con la diferenciación de las espermátidas hasta la fase acrosomal. La fase estacionaria del crecimiento testicular coincidió con una disminución en el contenido de CaM y con la aparición de espermatozoides en el epitelio germinal.

La evolución de los espermatocitos a través de la primera profase meiótica se asoció con un aumento en el contenido de CaM. Los espermatocitos en estadio de paquiteno mostraron los niveles más altos de esta proteína. La relación proteína total/CaM fue similar en los espermatocitos y espermátides tempranas. Los espermatozoides del epidídimo y la población de espermatogonias-espermatocitos leptoteno, mostraron el contenido más bajo de calmodulina.

Las proteínas blanco de CaM en la población de espermatogonias y células de Sertoli, fueron cualitativa y cuantitativamente diferentes a las detectadas en los espermatocitos y espermátidas. Sin embargo, estos dos tipos celulares mostraron un perfil semejante de proteínas aceptoras de Ca^{2+}-CaM, aunque el nivel fué más alto en los espermatocitos paquiteno.

Las proteínas aceptoras de CaM dependientes de Ca^{2+}, de la

matriz nuclear y del citosol de los espermatocitos paquiteno, se purificaron por cromatografía de afinidad y se determinó la presencia de proteín-cinasas dependientes de Ca^{2+}-CaM.

Los espermatocitos primarios presentaron tres tipos de cinasas de proteínas: la cinasa de caseína tipo I, localizada tanto en el núcleo como en el citoplasma y dos cinasas dependientes de Ca^{2+}-calmodulina. La proteín-cinasa tipo II, localizada en el citosol y la cinasa tipo I localizada en la matriz nuclear. Dos sustratos fosforilables, además de la proteín-cinasa II, posiblemente relacionados con el citoesqueleto, fueron purificados simultáneamente del citosol de los espermatocitos primarios.

La caracterización del sistema CaM-proteínas blanco se estudió también en el espermatozoide durante la capacitación y la reacción acrosomal. La expresión de la reacción acrosomal se correlacionó con cambios en la localización de CaM. Después de la reacción acrosomal, CaM se localizó en la región postecuatorial, involucrada en la interacción con el óvulo. La purificación de las membranas liberadas durante la reacción acrosomal, así como de las cabezas de los espermatozoides que ya habían sufrido la reacción acrosomal, permitió la identificación de las proteínas capaces de interaccionar con CaM en estas regiones del espermatozoide. Se demostró la presencia de proteínas que podía unir a CaM en forma dependiente e independiente de Ca^{2+}, tanto en las vesículas derivadas de la reacción acrosomal como en los restos membranales que quedan en la región subecuatorial y en la teca perinuclear de los espermatozoides que habían sufrido la reacción acrosomal.

Se discute la posibilidad de que la expresión genética en los espermatocitos y espermátidas pudiera ser controlada a través de las proteín-cinasas activadas por Ca^{2+}-CaM. También se discute el papel de CaM en el control del citoesqueleto de las células germinales en diferenciación y en el mantenimiento de dominios bien definidos en el espermatozoide.

INDICE

INTRODUCCION

I Ca^{2+}, calmodulina y el acoplamiento estímulo-respuesta

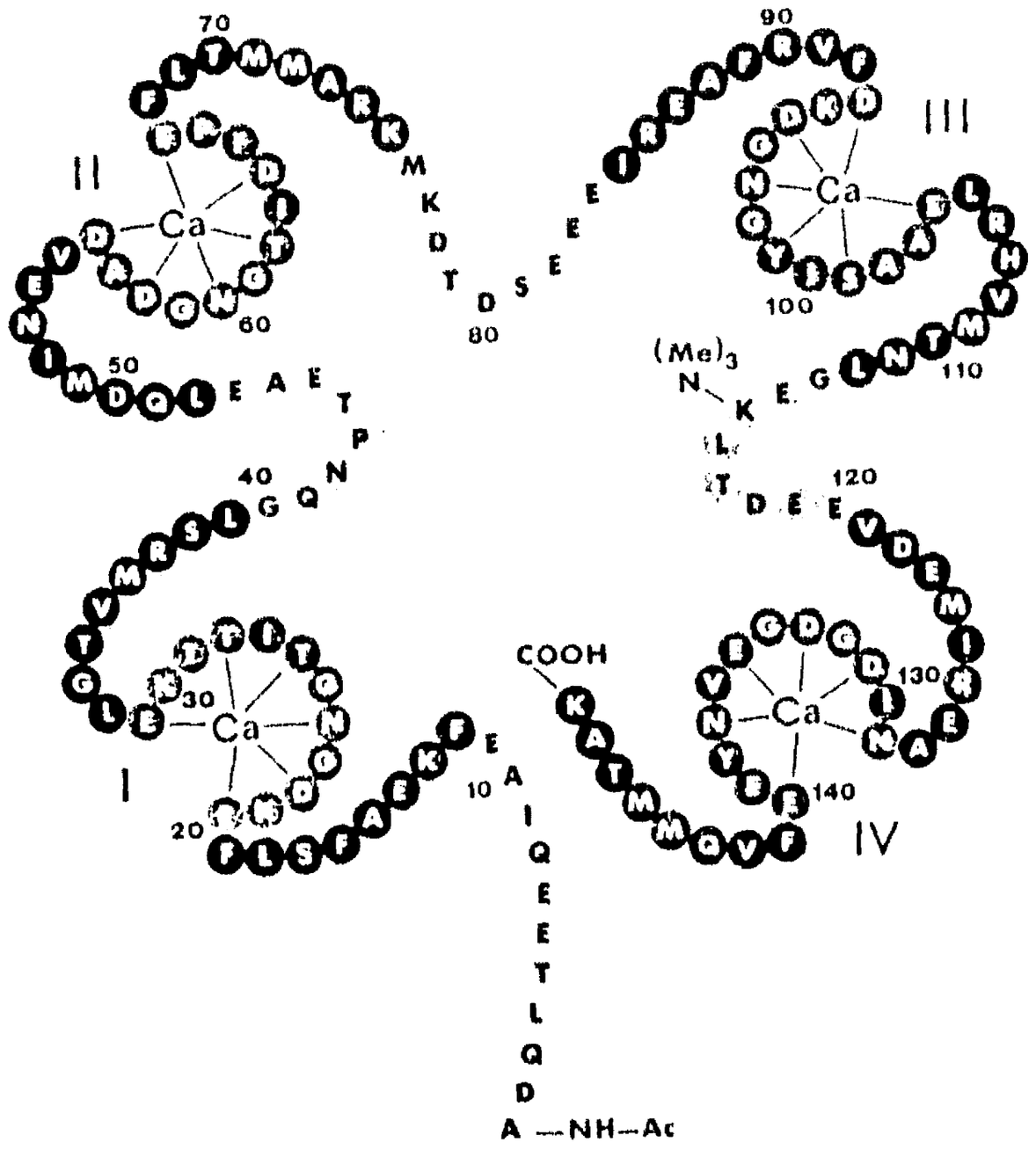
Aunque desde 1883 Sidney Ringer había demostrado el papel del calcio en la contracción muscular, fue hasta 1957 que Hodgkin y Keynes demostraron la existencia de proteínas receptoras de calcio localizadas tanto en células musculares como no musculares (revisado por Campbell, 1983). En 1968, Ebashi y Endo y en 1971, Greasser y Gergely, fueron los primeros en purificar una proteína intracelular capaz de unir calcio y en definir su papel como mediadora en una función calcio-dependiente: la troponina C era la proteína a la cual se unía el calcio y la que mediaba los efectos de este ión en la contracción muscular.

La demostración de que cambios transitorios en la concentración intracelular de Ca^{2+} podían disparar funciones celulares diversas, algunas muy complejas, como la proliferación celular, subrayó la necesidad de identificar el mecanismo de acoplamiento entre las señales externas que inducen estos procesos, el incremento en la concentración de Ca^{2+} y las proteínas responsables de la respuesta biológica final.

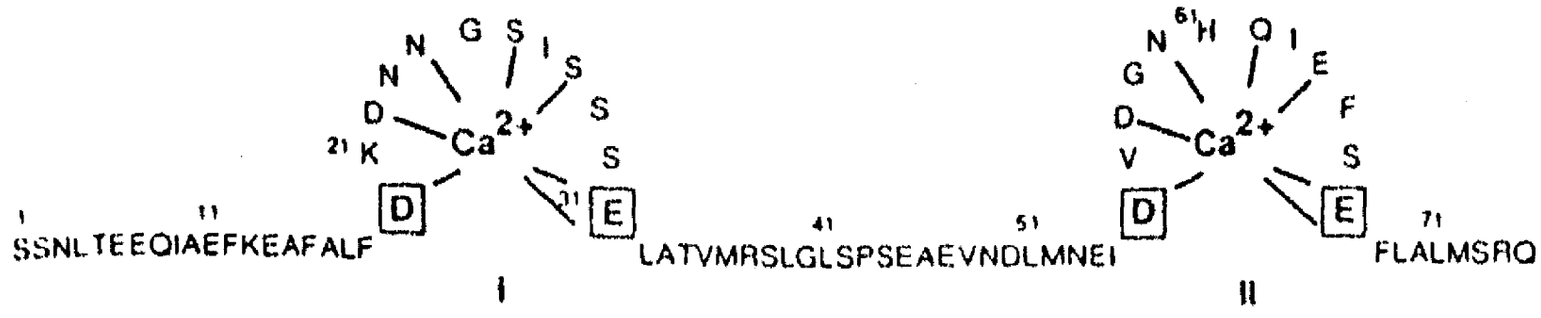
En 1970, dos grupos de trabajo independientes, el de Cheung y el de Kakiuchi y colaboradores reportaron que el cerebro contenía una proteína capaz de unir Ca^{2+} y de estimular la actividad de la fosfodiesterasa del AMPc. Incialmente, esta proteína recibió el nombre de receptor dependiente de Ca^{2+} (CDR) y posteriormente se denominó calmodulina (CaM). Wolff y Siegel reportaron su purificación en 1972 y Smoake y colaboradores, en 1974, fueron los primeros en observar las variaciones en el contenido de CaM de diversos tejidos durante el desarrollo. Posteriormente se demostró que CaM es una proteína ubicua, altamente conservada a lo largo de la escala filogenética y como la proteína aceptora de Ca^{2+} más abundante en células no musculares (Cheung, 1980; Means y Dedman, 1980).

CaM es una proteína constituída por 148 aminoácidos, con un peso molecular de 16 700 daltones, con un alto contenido de aminoácidos acídicos, lo que le confiere un pI de 4. Puede unir cuatro átomos de Ca^{2+} en dominios que presentan una conformación de hélice-asa-hélice conocida como EF, que se repiten cuatro veces. La porción NH_2 terminal contiene a los dominios I y II y la mitad COO^-

Fig. 1 A) Estructura primaria de la calmodulina bovina aislada de cerebro. Tomado de Watterson *et al.*, (1980). J. Biol. Chem. 255:462-475.



B) Secuencia de aminoácidos de los dominios que unen Ca^{2+}. Tomado de Geiser *et al.*, (1991). Cell 65:949-959.



alberga a los dominios III y IV (Fig. 1 A). Las asas a las que se une el Ca^{2+} están cargadas negativamente y están constituídas por doce aminoácidos, seis de los cuales participan en la formación de complejos multidentados con calcio. Los aminoácidos que ocupan las posiciones 1, 3, 5, 7 y 9 del asa contribuyen con cinco de los siete ligandos de oxígeno que coordinan al calcio. El aminoácido que ocupa la posición 12, generalmente glutámico, contribuye con el 6º y 7º ligandos. Aspártico o asparagina ocupan las posiciones 1, 3 y 5 (Babu y cols., 1988) (Fig. 1B). En ausencia de otras proteínas o de fármacos, se observa cooperatividad positiva entre los cuatro sitios que unen Ca^{2+} y que la afinidad de los dominios III y IV de la mitad COO^- es mayor a la de los dominios I y II, lo que define su orden de saturación (Cox, 1984). En presencia de proteínas o drogas que se unen a CaM, la afinidad de los cuatro sitios por Ca^{2+} es muy semejante y se comportan como una unidad (Vogel y cols., 1990; Weinstein y Mehler, 1994).

Conformación de CaM

CaM puede considerarse como una proteína simétrica con dos lóbulos conectados por un segmento central con conformación alfa hélice. Cada lóbulo presenta dos estructuras hélice-asa-hélice, puede unir dos átomos de Ca^{2+} y constituye un dominio hidrofóbico. La longitud de la molécula es de 65 Å (Fig. 2).

CaM presenta siete regiones con alfa hélice, en los aminoácidos 7-19, 29-40, 46-55, 65-92, 102-112, 119-128 y 138-148. Las dos asas que unen Ca^{2+} de cada dominio son antiparalelas y dan lugar a pequeñas regiones, de tres aminoácidos, con conformación ß-plegada (Babu y cols., 1988). La cooperatividad positiva entre los sitios da como resultado un cambio conformacional muy acentuado después de que el segundo calcio se ha unido, aumenta el contenido de alfa hélice y se exponen los dominios hidrofóbicos (Klee, 1977; Richman y Klee, 1979; LaPorte y cols., 1980; Crouch y Klee, 1980; Tanaka e Hidaka, 1980; Gopalakrishna y Anderson, 1982).

La estructura alargada que CaM presenta en ausencia de proteínas aceptoras, cambia a una conformación más compacta cuando se asocia a alguna de sus proteínas blanco, debido a la flexibilidad y a la longitud de la hélice central, ocho vueltas, que permite el acercamiento de los dos dominios hidrofóbicos (Babu y cols., 1985; O'Neil y cols., 1990; Meador y cols., 1992, 1993).

Fig. 2. Conformación de Calmodulina. Par estereoscópico. Tomado de Babu *et al.*, (1988). En "Molecular Aspects of Cellular Regulation", Vol. 5. Calmodulin. Editado por P. Cohen y C. B. Klee. Elsevier Publishing Co. pp83-90.

Azúl obscuro: arginina y lisina. Rojo: aspártico y glutámico. Amarillo:histidina, tirosina, serina y treonina.
Morado: asparagina y glutamina. Azúl claro: glicina, alanina y prolina. Verde: metionina, valina, leucina, isoleucina y fenilalanina.

Proteínas blanco de CaM

La actividad de más de veinte enzimas, incluyendo a proteínas cinasas y fosfatasas de proteínas, es estimulada por CaM en forma Ca^{2+}-dependiente (revisado por Cheung, 1980; Means y Dedman, 1980; James y cols., 1995). Se ha demostrado que el complejo Ca^{2+}-CaM se une a las proteínas que tienen secuencias de aminoácidos polares y básicos alternando con aminoácidos hidrofóbicos capaces de constituir regiones con alfa hélice. Los aminoácidos hidrofóbicos y los residuos básicos y polares ocupan posiciones opuestas en la hélice (Fig. 3). La unión del complejo Ca^{2+}-CaM con la proteína blanco se mantiene por interacciones con los dos dominios hidrofóbicos de CaM y por interacciones electrostáticas (O'Neil y DeGrado, 1990; Meador y cols., 1992, 1993).

Se ha demostrado que ocho de las nueve metioninas presentes en CaM forman parte de los dominios hidrofóbicos y están directamente relacionadas con la actividad de Ca^{2+}-CaM (Vogel y cols., 1990) (Fig. 4). Aunque los residuos de lisina, histidina y tirosina se encuentran expuestos en el complejo Ca^{2+}-CaM, parecen estar lejos de los dominios hidrofóbicos y no intervienen en la interacción con otras proteínas o con compuestos hidrofóbicos.

Algunas de las proteínas blanco, como la fosfodiesterasa o la fosforilasa b cinasa, requieren interaccionar con toda la molécula, tanto la forma parcialmente saturada con tres átomos de Ca^{2+}, como la completamente saturada, CaM-Ca^{2+}_4, son las que participan en este proceso (Cox, 1984). Sin embargo se ha demostrado que algunas proteínas son capaces de ser activadas por fragmentos de CaM, ya sea la mitad COO- o la mitad NH_2, (Kuznicki y cols., 1981; Newton y cols., 1984; Ni y Klee, 1985).

Por otra parte también se ha demostrado que algunas proteínas pueden unir y aún ser activadas por CaM en ausencia de Ca^{2+} (Kilhoffer y cols., 1983). Se ha sugerido que las proteínas que no requieren Ca^{2+} para asociarse a CaM, posiblemente estén relacionadas con la distribución intracelular de CaM y con la regulación de otras proteínas aceptoras de CaM dependientes de Ca^{2+} (Andreasen y cols., 1983; Cimier y cols., 1985; Kilhoffer y cols., 1983; Aitken y cols., 1988).

Proteínas cinasas activadas por Ca^{2+}-CaM (CaMK)

La fosforilación de proteínas dependiente de Ca^{2+}-CaM es

Fig. 3. Representación de la estructura helicoidal reconocida por calmodulina en las proteínas blanco. Aminoácidos polares básicos alternados con aminoácidos hidrofóbicos, de tal manra que ocupan posiciones opuestas en la hélice. a) péptido modelo. b) espectrina. Tomado de O'Neil y De Grado (1990). TIBS 15:59-64.

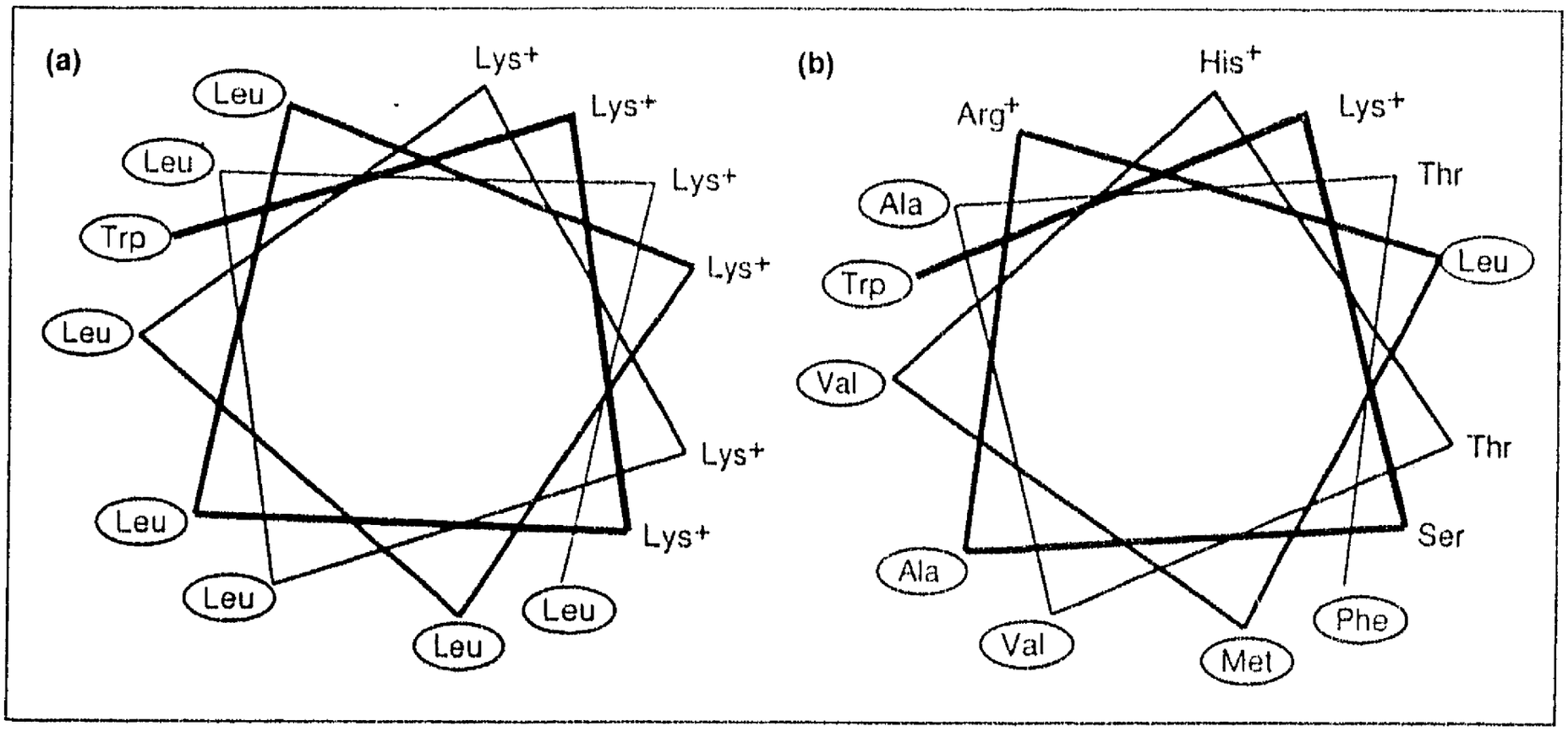
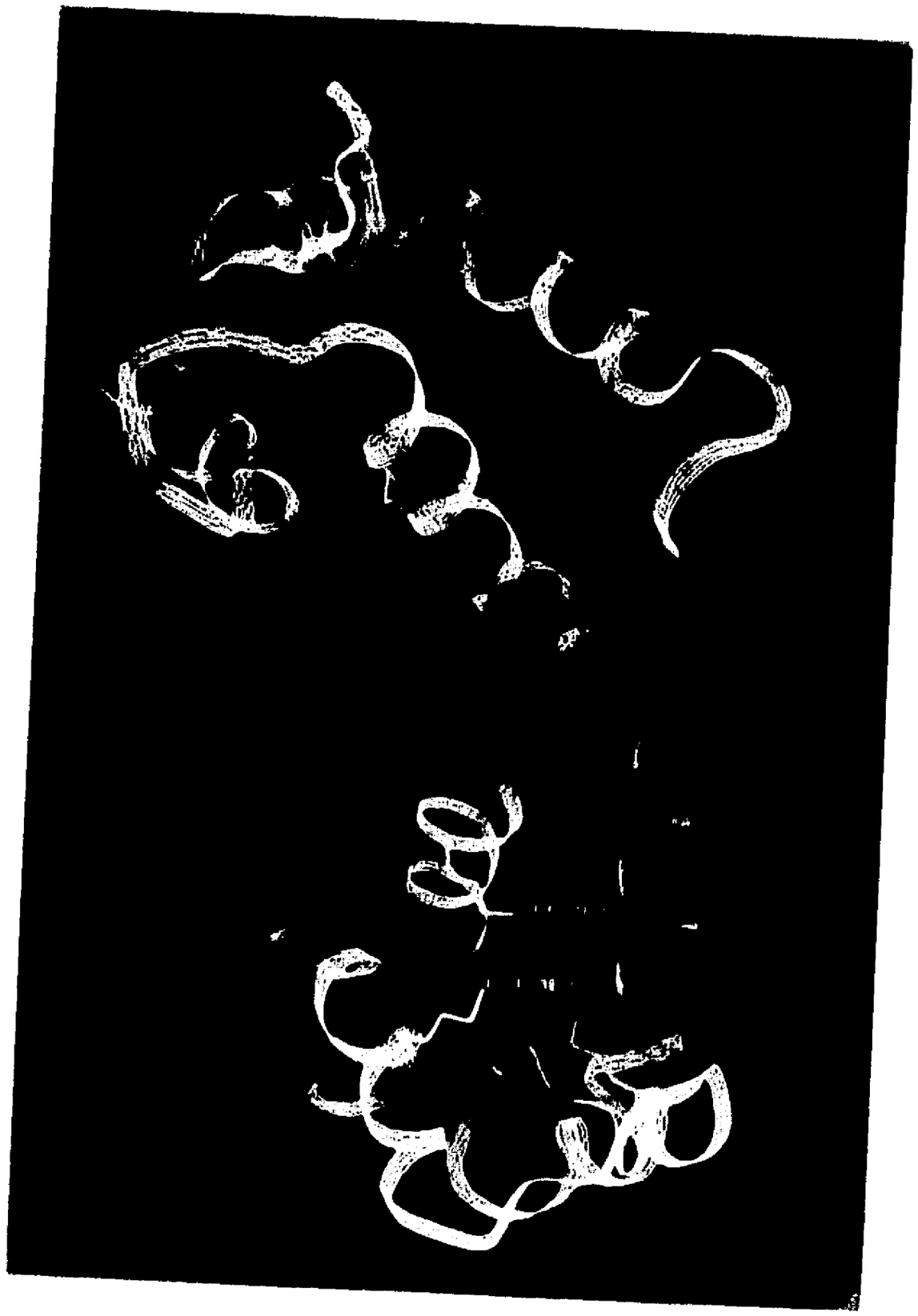


Fig. 4. Estructura de calmodulina asociada a una proteína blanco. La calmodulina está representada en color amarillo, los residuos de metionina de los dominios hidrofóbicos están señalados en color verde. En color azul se indica la posición de las metioninas 171, 124 y 144. Tomado de O'Neil y De Grado (1990). TIBS 15: 59-64



realizada al menos por ocho proteínas cinasas diferentes. Tres de ellas, la fosforilasa b cinasa (Werth y cols., 1982), la cinasa de la cadena ligera de la miosina (MLCK) (Walsh y cols., 1979) y la CaMK III (Nairn y cols., 1985; Nairn y Palfrey, 1987) fosforilan sustratos específicos: fosforilasa b, la cadena ligera de la miosina y el factor de elongación 2, respectivamente. CaMK I (DeRemer y cols., 1992, 1992a) y CaMK V (Mochizuki y cols., 1993), fosforilan a la sinapsina I en el sitio 1 y a la proteína que une AMPc (CREB). Estas dos cinasas y la CaMK IV son reguladas por fosforilación a través de otra proteína cinasa activada también por Ca^{2+}-CaM (Selbert y cols., 1995). CaMK IV (Miyano y cols., 1992) y CaMK II (revisado por Cohen, 1988) son proteínas capaces de fosforilar numerosos sustratos.

Se ha propuesto que las proteínas activadas por Ca^{2+}-CaM poseen una secuencia inhibitoria interna, lejana al sitio catalítico pero superpuesta a la región donde se une Ca^{2+}-CaM. Este efecto inhibitorio puede ser abolido por la unión de Ca^{2+}-CaM, por la eliminación de esos aminoácidos mediante una proteólisis limitada o por fosforilación. En los dos últimos casos, se produciría una enzima independiente de Ca^{2+}-CaM (revisado por James y cols., 1995).

II Calmodulina y proliferación celular

La participación de CaM como mediadora de Ca^{2+} en el control de la proliferación celular ha sido demostrada por los resultados obtenidos con enfoques experimentales diferentes:

-Experimentos *in vivo* utilizando animales con hepatectomía parcial demostraron que la síntesis de DNA y la proliferación de las células residuales era precedida por un aumento transitorio en el contenido de Ca^{2+} y de CaM. Si cualquiera de estos eventos era bloqueado, por paratiroidectomía o por administración de drogas antiCaM, no se observaba la recuperación de la masa hepática (MacManus y cols., 1981; Piñol y cols., 1988). También se demostró que la actividad de la DNA polimerasa y era estimulada por Ca^{2+}-CaM y no se presentaba en animales tratados con agentes antiCaM (López-Girona y cols., 1992, 1995).

-Experimentos *in vitro* con diferentes tipos celulares demostraron que el inicio de la fase S y la mitosis eran bloqueados

por la adición de compuestos antiCaM (Chafouleas y cols., 1982).

-Empleando levaduras mutantes se demostró que la pérdida del gene que codifica a CaM produce células no viables para la mitosis, demostrando así que CaM es una proteína esencial para la proliferación celular. (Davis, 1986; Geiser y cols., 1988).

-La posibilidad de controlar la expresión del gene que codifica a CaM, y por lo tanto el nivel intracelular de la proteína, permitió definir que bajos niveles de CaM producen una segregación anormal de los cromosomas y pérdida del material genético (Takeda y Yamamoto, 1987; Ohya y Anraku, 1989), en tanto que niveles altos podían acortar la fase G_1 (Rasmussen y Means, 1987, 1989).

Concentración de calmodulina

El nivel intracelular de CaM se duplica en la transición G_1/S (Chafouleas y cols., 1982), pero la relación CaM/proteína total no se modifica (Veigl y cols., 1984). Niveles elevados de CaM pueden acelerar el ciclo celular a través de disminuir la duración de la fase G_1 (Rasmussen y Means, 1987, 1989). En células inducidas a multiplicarse, por ejemplo después de hepatectomía parcial, el contenido total de CaM se incrementa en forma transitoria, observándose que el nivel nuclear de CaM se eleva porque tan pronto es sintetizada, CaM migra al núcleo (Serratosa y cols., 1988; Pujol y cols., 1989).

Proteínas blanco de CaM involucradas en la división celular

Se ha demostrado que CaM participa en el rompimiento de membrana nuclear durante la profase (Baitinger y cols., 1990), en la transición metafase/anafase (Lorca y cols., 1991, 1993, 1994; Morin y cols., 1994) y en el control de la expresión de los genes de las proteínas cinasas dependientes de ciclinas, de las ciclinas y de las enzimas pre-replicativas (Colomer y cols., 1994), a través de la fosforilación de proteínas efectoras catalizada por la CaMK II dendiente de Ca^{2+}-CaM.

Localización de CaM

En células en división, CaM se localiza en el huso mitótico, especialmente en los microtúbulos del cinetocoro (Welsh y cols.,

1978; Marcum y cols., 1978; Vantard y cols., 1985). Durante la interfase, CaM se localiza en el citoplasma asociada a las fibras de tensión, a las mitocondrias, a los centriolos (Pardue y cols., 1983; Dedman y cols., 1978) y en el núcleo de diversos órganos (Harper y cols., 1980). Se ha sugerido que los cambios localizados en la concentración de Ca^{2+} pueden modificar la distribución intracelular de CaM. También se ha demostrado que CaM es transportada al núcleo mediante un acarreador y que este transporte no requiere de energía (Pruschy y cols., 1994).

En diversos tejidos normales la mayor parte del contenido intracelular de CaM se recupera en la fracción citosólica y un porcentaje pequeño se encuentra asociado a la porción particulada (Van Eldik y Burgess, 1983).

III Diferenciación del epitelio germinal testicular

El epitelio germinal testicular muestra cambios cualitativos y cuantitativos muy importantes durante el desarrollo. En la rata recién nacida, los túbulos seminíferos están constituídos por gonocitos y células de soporte que darán origen a las espermatogonias y a las células de Sertoli, respectivamente. Las espermatogonias tipo A pueden reconocerse desde los 4 días de edad. Las espermatogonias se multiplican por mitosis y su diferenciación da origen a las espermatogonias intermedias, que a su vez producen las espermatogonias tipo B (Clermont y Perey, 1957). Las espermatogonias tipo B dan origen a los espermatocitos, que se multiplicarán por meiosis.

La profase de la primera división meiótica tiene una duración aproximada de 12-14 días. Los espermatocitos en estadio preleptoteno aparecen a los 12 días de edad, alcanzan el estadio zigoteno a los 14 días; el estadio paquiteno se inicia a los 15 días y se prolonga hasta los 24 días de edad. Las siguientes etapas de la primera y segunda división meiótica transcurren rápidamente en algunas horas. Las espermátidas aparecen a los 26 días de edad, no se dividen y su diferenciación requiere de un tiempo prolongado, ya que los espermatozoides aparecen a los 45 días de edad (Clemont y Perey, 1957).

La densidad que alcanzan los distintos tipos celulares en el epitelio germinal se modifica durante el desarrollo. En la rata Sprague Dawley las espermatogonias alcanzan la máxima densidad

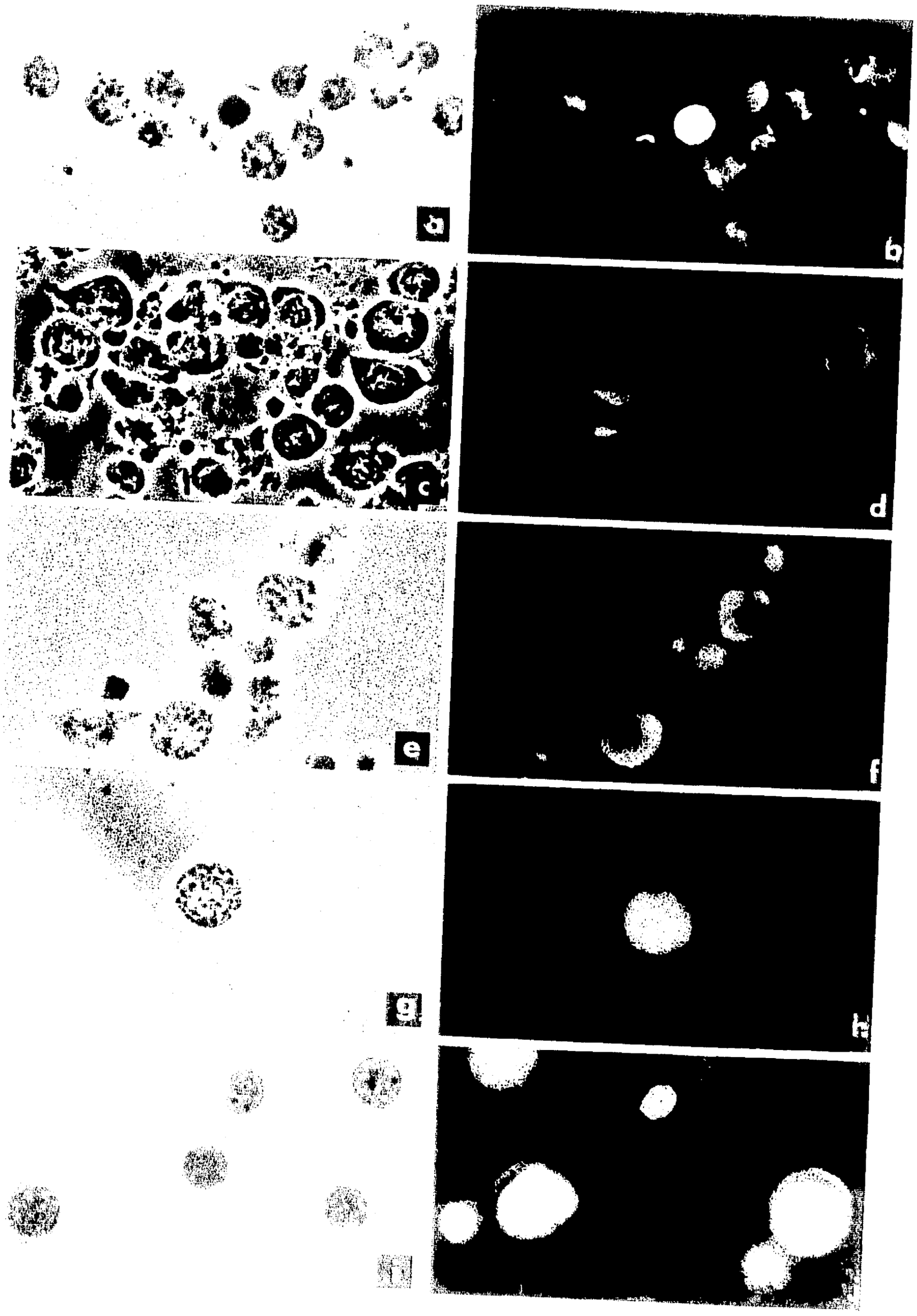
entre los 10 - 20 días de edad. Los espermatocitos primarios son el tipo celular predominante a los 25 días y las espermátidas alcanzan su máxima densidad a los 40 días de edad y su proporción dentro del túbulo permanece constante durante la vida adulta (Zhengwei y cols., 1990).

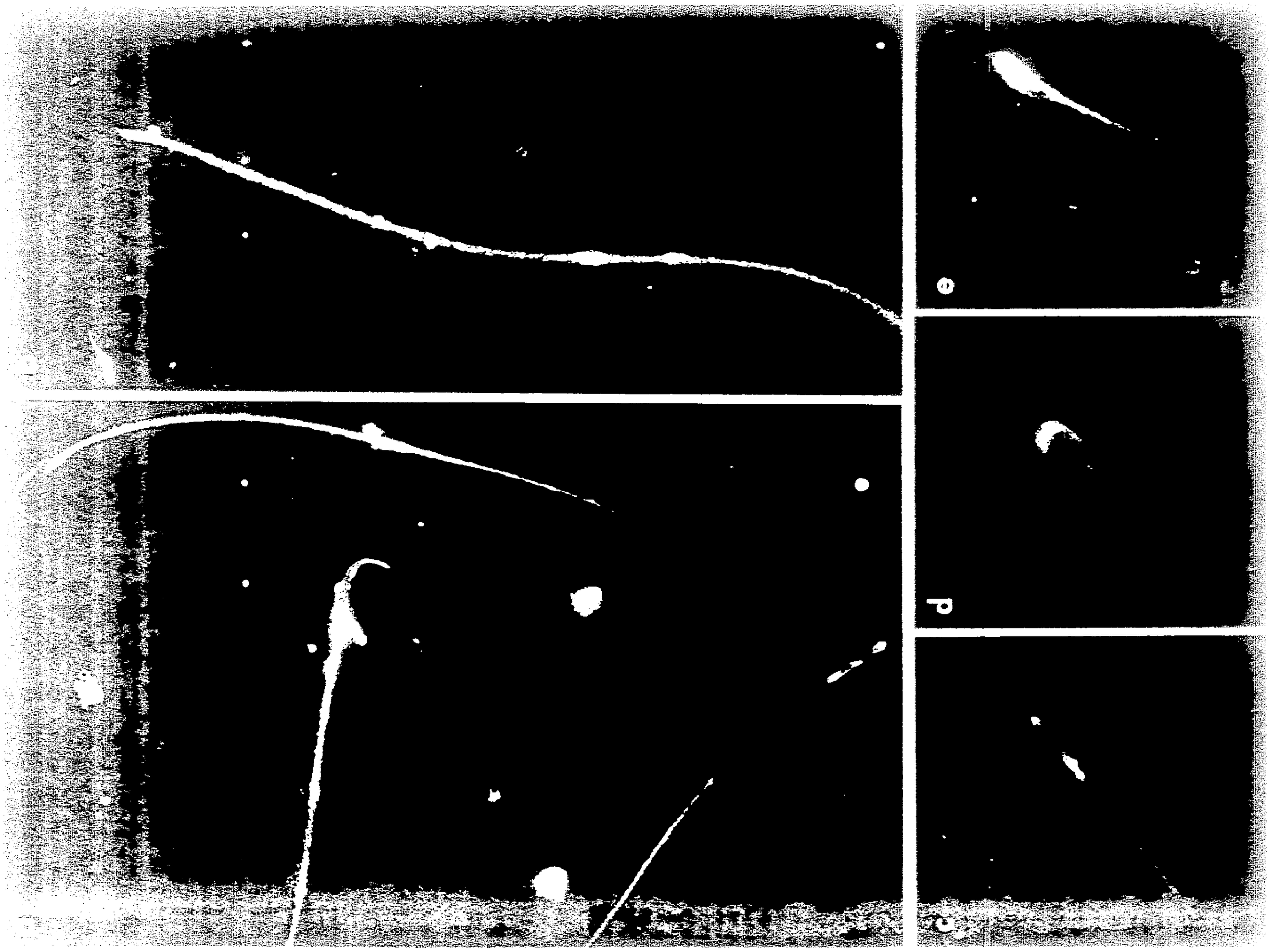
Desarrollo testicular y CaM

A diferencia de otros tejidos, la relación CaM/proteína total aumenta durante el desarrollo testicular (Smoake y cols., 1974). La fase de incremento coincide con la etapa de aparición de espermatocitos y de espermátidas (Smoake y cols., 1974; Lagacè y cols., 1981; Trejo y cols., 1985; Slaughter y cols., 1987). Se observó que los espermatocitos y espermátidas aislados de testículo inmaduro presentaban niveles elevados del RNA mensajero de esta proteína (Trejo y cols., 1985). Estudios posteriores demostraron que las células espermatogénicas poseen tres genes que codifican a CaM y se sugirió que las variaciones en el contenido de CaM podrían estar relacionadas con la expresión diferencial de los genes que la codifican (Slaughter y cols., 1987; Slaughter y Means, 1987, 1989).

Mediante técnicas de inmunofluorescencia indirecta se observó que la localización de CaM se modificaba dependiendo del estadio alcanzado por las células germinales. En los espermatocitos en el estadio preleptoteno la inmunofluorescencia se localizaba principalmente en el núcleo (Fig. 5b). En los espermatocitos en estadio paquiteno temprano, CaM se distribuía en el citoplasma (Fig. 5d), pero en la fase tardía de este estadio (Fig. 5f) y en el estadio de diploteno (Fig. 5h), nuevamente se concentraba en el núcleo. CaM permaneció asociada al núcleo después de la meiosis, las espermátides tempranas, fase Golgi, mostraron una clara señal inmunofluorescente nuclear (Fig. 5j). Durante estadios posteriores de la espermiogénesis se observó la asociación de CaM a diferentes estructuras. Espermátidas en la fase acrosomal y en la de maduración presentaron a CaM localizada en el citoplasma, asociada al flagelo en formación y al acrosoma. En el espermatozoide aislado de epidídimo de rata adulta, se observó la distribución de CaM a lo largo del flagelo y en la gota citoplásmica (Fig. 6a y b). También se observó que CaM parecía concentrarse en la punta del acrosoma y en el núcleo del espermatozoide (Fig. 6c,d,e) (Lagacé y cols., 1981; Trejo y cols., 1985; Moriya y cols., 1994).

Fig.5. Inmunolocalización de vimentina en las células germinales.




a
b
c

CaM y espermatozoide

La demostración de que los espermatozoides de vertebrados e invertebrados contenían CaM (Jones y cols., 1978), localizada en el acrosoma, en la región ecuatorial-subecuatorial de la cabeza y en el flagelo (Jones y cols., 1980; Camatini y cols., 1986), sugirieron la participación de CaM en los procesos Ca^{2+}-dependientes del espermatozoide.

Aunque varios agentes con actividad anti-CaM, como trifluoroperazina (TFP) y W-7, han sido empleados para estudiar el papel de CaM en la reacción acrosomal, en la movilidad flagelar y en la fertilización, los resultados obtenidos por varios autores son contradictorios. A dosis bajas y empleando espermatozoides de conejo, TFP y W-7 tuvieron un efecto inhibitorio de la reacción acrosomal y la fertilización, en tanto que a dosis elevadas aceleraron la liberación del contenido acrosomal (Lenz y Cormier, 1982). En el espermatozoide de erizo de mar, W-7 inhibió la reacción acrosomal (Sano 1983), pero en el espermatozoide de cuyo tuvo un efecto inductor (Nagae y Srivastava, 1986). La inespecificidad de estos compuestos y sus efectos sobre la permeabilidad de la membrana, descartan a CaM como el blanco exclusivo de los agentes usados y por lo tanto como causa única de los efectos producidos. Por otra parte, los espermatozoides de mamífero requieren de un proceso capacitante para expresar la reacción acrosomal, durante el cual la permeabilidad de la membrana al Ca^{2+} aumenta. Los resultados observados podrían reflejar los efectos de las drogas anti-CaM sobre la capacitación y/o sobre la fusión de membranas y la liberación del contenido acrosomal.

PLANTEAMIENTO DEL PROBLEMA

A diferencia de otros tejidos que mantienen la relación proteína total/CaM constante durante el desarrollo y de tejidos experimentalmente activados a proliferar, que presentan un incremento transitorio en el contenido de CaM, el testículo de la rata presentó una elevación sostenida en la relación proteína total/CaM.

La fase de incremento en el contenido testicular de CaM se presentó entre los 20 y los 35 días de edad, período en el cual los espermatocitos primarios completan la división meiótica y aparecen las espermátides.

Se ha observado que altos niveles de CaM aceleran la proliferación celular, sin embargo no se determinó si la velocidad de crecimiento del testículo estaba relacionada con el contenido de CaM. Tampoco se determinó la contribución de cada tipo celular al contenido total de CaM.

En otros sistemas celulares se ha demostrado que existe una síntesis coordinada de CaM y de sus proteínas blanco, que se traduce en cambios funcionales del sistema Ca^{2+}-CaM-proteínas aceptoras. Esta situación no fue definida en el testículo en desarrollo ni en las células germinales en los distintos estadios de diferenciación.

La migración de CaM al núcleo de los espermatocitos primarios en estadios tardíos de la primera profase meiótica, semejante a la migración de CaM observada en células activadas a dividirse, sugiere la participación de CaM en los procesos cromosómicos que se realizan en los últimos estadios de la primera profase meiótica, por lo que se requiere identificar a las proteínas capaces de unir a CaM en el núcleo de los espermatocitos primarios y estudiar su función.

El efecto inhibidor o estimulador de la reacción acrosomal observado con drogas anti-CaM como TFP o W-7, parecen ser debidos a la perturbación de la permeabilidad de la membrana del espermatozoide y no puede ser explicado considerando los efectos de estos compuestos sobre CaM. La localización de CaM en el acrosoma, en la gota citoplásmica y en el flagelo del espermatozoide, sugiere la existencia de proteínas aceptoras de CaM relacionadas a la reacción acrosomal y al movimiento flagelar. La dependencia de Ca^{2+} de la reacción acrosomal y los cambios en la permeabilidad al Ca^{2+} que se presentan durante la capacitación y la reacción acrosomal

del espermatozoide, podrían modificar la localización de CaM y poner de manifiesto proteínas blanco de CaM dependientes de Ca^{2+}. relacionadas con la fusión de membranas que se requiere para liberar el contenido acrosomal.

OBJETIVOS

Objetivo general

Determinar las funciones que realiza CaM durante la diferenciación del epitelio germinal mediante la identificación de sus proteinas blanco en las células germinales aisladas.

Objetivos específicos

1.- Determinar si el contenido y distribución subcelular de CaM así como de sus proteínas blanco están relacionados con la velocidad del crecimiento del órgano.

2.- Determinar la contribución de cada tipo celular al contenido total de CaM y definir si el estadio de diferenciación alcanzado por las células germinales se relaciona con la presencia de proteinas aceptoras específicas de CaM.

3.- Aislar y purificar las proteínas blanco de CaM en los espermatocitos primarios.

4,. Definir si los espermatocitos poseen actividad de proteína cinasa activada por Ca^{2+}-CaM.

5.- Definir si durante la capacitación y reacción acrosomal del espermatozoide se pueden identificar proteínas aceptoras de CaM que participen en esos procesos.

RESULTADOS

Para alcanzar el primer objetivo de esta tesis, **determinar la relación entre el contenido de CaM y la velocidad de crecimiento testicular,** se registró la evolución del peso del testículo, se determinó la relación mg proteína/g tejido, el contenido de agua, se cuantificó el nivel de CaM y se determinó la relación proteína total /CaM. El número de animales sacrificados para cada edad, varió entre 50 y 4 ratas macho. El peso testicular se obtuvo dividiendo el peso total del tejido obtenido entre el número de testículos disecados. El contenido de agua se determinó tomando el peso húmedo y el peso seco de 2 a 4 testículos a cada edad. El contenido de proteína y el nivel de CaM se determinaron en alícuotas del homogeneizado total testicular a las diferentes edades, de 5 a 60 días, con intervalos de 2 a 5 días. Como medio de homogeneización se empleó una solución salina amortiguada con boratos que incluyó EGTA y SDS. El homogeneizado fue sonicado durante 2 min a 4°C para asegurar la completa extracción de CaM.

Para definir si la velocidad de crecimiento modificaba la distribución subcelular de CaM, se analizó el contenido de esta proteína en seis fracciones subcelulares obtenidas a partir del homogeneizado total de testículo de rata adulta y de testículo de rata de 20 días, correspondientes a la meseta y a la fase de crecimiento exponencial del testículo. Cada una de las fracciones fué resuspendida con la solución salina y sonicada de la forma descrita para el homogeneizado total.

El contenido de CaM fué determinado por radioinmunoanálisis utilizando como estándar CaM purificada de testículo de toro marcada con ^{125}-I. Los anticuerpos anti-CaM bovina fueron inducidos en borrego y purificados por cromatografía de afinidad. Los resultados indican que la velocidad del crecimiento testicular está relacionada con el contenido de CaM, durante la fase de crecimiento exponencial el contenido de CaM aumenta. Sin embargo, la velocidad de crecimiento no modificó la distribución subcelular. El 80 % de la CaM total se recupera en el citosol y la pastilla nuclear.

Para definir si al igual que en otros tipos celulares, las variaciones en el contenido de CaM se asociaban con cambios en las proteinas aceptoras, alícuotas de los homogeneizados de testículo de rata de 5, 20 y 40 días de edad fueron separadas por electroforesis y la presencia de las proteinas aceptoras de CaM dependientes de Ca^{2+} fue determinada por la técnica de overlay

utilizando ^{125}I-CaM y autoradiografía.

Los resultados señalan que durante la fase de crecimiento exponencial, a los 20 días de edad, se observan niveles más altos de proteínas capaces de unir a CaM en forma dependiente de Ca^{2+}, pero no se apreciaron cambios cualitativos significativos.

Por otra parte la velocidad de crecimiento no modificó el patrón de distribución subcelular de las proteínas aceptoras de CaM, ya que a 13 o 20 días de edad el perfil de proteínas capaces de unir a CaM en forma dpendiente de Ca^{2+} fué muy similar al observado en la edad adulta. Varias proteínas de peso molecular alto predominaron en las fracciones membranales más ligeras y en el citosol. Las proteínas de bajo peso molecular, predominaron en la pastilla nuclear obtenida a 770 x g.

Para alcanzar el segundo objetivo, **determinar la contribución de cada tipo celular al contenido total de CaM y definir si las proteinas aceptoras de CaM se modificaban durante la diferenciación de las células germinales** , se obtuvieron poblaciones enriquecidas en espermatogonias y células de Sertoli, en espermatocitos primarios y en espermátidas, a partir de testículo de rata de 12, 21 y 38 días de edad, respectivamente y se determinó su contenido de CaM.

Los espermatocitos primarios mostraron un contenido mayor de CaM, expresado como pg CaM/célula, que el observado en las espermátidas. Sin embargo, la relación proteína total/CaM fue semejante en ambos tipos celulares. Los espermatozoides, las espermatogonias y las células de Sertoli presentaron la relación pg CaM/célula más baja.

Las espermatogonias y las células de Sertoli mostraron diferencias cualitativas y cuantitativas en las proteínas aceptoras de CaM respecto a las células germinales en meiosis y postmeióticas. Sin embargo, los espermatocitos y espermátidas presentaron un patrón semejante de proteínas capaces de unir a CaM en forma Ca^{2+}-dependiente.

Para identificar las proteínas nucleares a las que se une CaM en estadíos tardíos de la meiosis, los núcleos de los espermatocitos primarios en estadio paquiteno tardío fueron purificados y la presencia de las proteínas aceptoras de CaM fue determinada tanto en el núcleo como en el citoplasma. En ambas fracciones se encontraron proteínas que unieron a CaM sólo en presencia de Ca^{2+}, pero a diferencia del citoplasma, el núcleo de los espermatocitos presentó proteínas de bajo peso molecular que no

requirieron Ca^{2+} para unir a CaM.

Los resultados se muestran a continuación en el trabajo "Characterization of the calmodulin-acceptor protein system in developing rat testis" sometido a consideración del comité editorial de la revista Molecular Reproduction and Development. Fecha de envío 2 de mayo de 1996 y registrado con el número 4456.

Characterization of the calmodulin-acceptor protein system in developing rat testis.

Raquel Trejo*[1,2], Rodney Turner[2], Graciela Delhumeau[3], and John R. Dedman[2,4].

[1]Unidad de Investigación Médica en Enfermedades Oncológicas. Hospital de Oncología. Instituto Mexicano del Seguro Social. Apdo. Postal 12-1060. México D.F. México 03000.

[2]Departments of Physiology, Cell Biology and Internal Medicine. University of Texas Medical School at Houston. Houston, Texas.

[3]Unidad de Investigación Médica en Genética Humana. Hospital de Pediatría. Instituto Mexicano del Seguro Social. México D.F.

[4]Present address: Department of Physiology and Biophysics. University of Cincinnati College of Medicine. Cincinnati, Ohio 45267-0576.

* To whom correspondence should be sent.
Telephone numbers (525) 627-6900 ext 4323 and 4204.
Fax number (525) 761-0952.

Running title: Calmodulin-binding proteins in rat testis

ABSTRACT

In order to characterize the calmodulin-acceptor protein system in male germinal cells, the specific content as well as the subcellular distribution of calmodulin (CaM) and Ca^{2+}-dependent calmodulin-binding proteins (CaMBP) were determined at several stages of rat testis development. Three specific rises in CaM were observed, the first between 5-10 days, the second one at 24 days and the third one at 32 days. The first CaM increase preceded the proliferation of Sertoli cells and spermatogonia. The second and third rises occurred during the exponential phase of testis growth and were paralleled by completion of meiosis and by differentiation of spermatids, respectively. A decrease in CaM content was observed at the plateau phase of testis growth when maturation of spermatids takes place and spermatozoa appear in the germinal epithelium. On cell basis, pachytene primary spermatocytes showed the highest CaM content. Sertoli cells plus spermatogonia population and epidydimal spermatozoa showed the lowest level. In immature and adult rat testis CaM and Ca^{2+}-CaMBP were mainly associated to nuclear and cytosol fractions. A similar pattern of Ca^{2+}-CaMBP was obsrved at several stages of testis development. However, the level was higher when germinal epithelium have differentiated up to pachytene stage. Purified nuclei from primary spermatocytes showed several Ca^{2+}-dependent CaMBP but also showed Ca^{2+}-independent CaMBP, whereas the cytosol only showed Ca^{2+}-dependent CaMBP.

Key words: calmodulin, calmodulin-binding proteins, rat testis development, spermatogenesis, pachytene spermatocytes.

ABSTRACT

Calmodulin (CaM), the ubiquitous Ca^{2+}-binding protein, has been involved in the control of cell cycle progression. Mitosis and chromosome segregation depend on adequate CaM level. Increased CaM content reduces the length of G_1, whereas low CaM level or CaM gene deletion causes abnormal chromosome segregation and non viable cells at mitosis. Changes in CaM-binding proteins (CaMBP), in coordination with increased CaM level, have been observed in cells induced to proliferate.

Although the testicular content of CaM increases during development, the contribution made by specific germ cells, the relationship between CaM content and growth rate, the identity of the effector proteins or CaMBP have not been defined. To accomplish this, testis CaM content was determined in developing rats from 5 to 60 days and in isolated germ cells at specific stages of differentiaton. The subcellular distribution of CaM and Ca^{2+}-dependent CaMBP was determined at the exponential and plateau phases of growth.

It was found that rat testis development was paralleled by three rises in CaM content. The first rise was observed between 5-10 days, coincident with spermatogonia and Sertoli cell proliferation. The second and the third increases occurred during the exponential phase of testicular growth. The second CaM increase occured at 24 days, coincident with the last stages of meiosis and spermatids appearance. The third rise in CaM was observed at 34 days when spermatids reach the acrosome phase. The plateau phase of growth, spermatid maturation and the appearance of spermatozoa were coincident with a decrease in CaM content. CaM content and Ca^{2+}-CaMBP were also studied in isolated spermatogonia, primary spermatocytes, spermatids and spermatozoa. Pachytene primary spermatocytes showed the highest CaM level, but the CaM/total protein ratio was similar in both spermatids and pachytene spermatocytes.

The differentiation of germinal cells was accompained by changes in CaM localization, in pachytene parimary spermatocytes and in early spermatids CaM was localized in the nucleus. To better identify the CaM targets, pachytene primary spermatocytes were fractionated and the Ca^{2+}-CaMBP from nuclear matrix and cytosol were purified by affinity chromatography. Our results suggests that primary spermatocytes present three types of protein kinases, the casein kinase I and two protein kinases stimulated by Ca^{2+}-CaM. Ca^{2+}-CaM kinase II was observed in the cytosol and the type I was

found in the nuclear matrix. However, the use of specific inhibitors and several substrates should be included to clearly define the type of kinases found.

In the sperm, CaM was localized in the acrosome and along the tail. However, the distribution of CaM changed during the acrosome reaction. CaM was released together with the acrosomal membrane vesicles. In acrosome reacted sperm, CaM was associated to the subequatorial region of the head, which has been involved in sperm-egg interaction. To define whether specific CaMBP were present in the acrosome and in the subequatorial region of the head, the vesicles released after the acrosomal reaction were recovered and the membrane material remaining on acrosome reacted sperm heads was solubilized to assay the ability to bind CaM with and without Ca^{2+}. Several Ca^{2+}-independent CaMBP were identified in the membrane vesicles released and in the membrane material recovered from acrosome reacted sperm heads, but not Ca^{2+}-dependent CaMBP.

The role(s) that CaM might play in germ cells is discussed.

INTRODUCTION

Evidence obtained under different experimental conditions indicates that calmodulin (CaM) is involved in the control of cell growth and differentiation. A wave in CaM level precedes the syntesis of DNA in cells induced to proliferate (MacManus *et al.*, 1981; Piñol et al., 1988). Cell cycle progression rate, specifically the lenght of G_1, appeared to be responsive to CaM concentration (Rasmussen and Means, 1987, 1989). Its localization in the mitotic spindle (Welsh *et al.*, 1978; Marcum *et al.*, 1978) and the requirement of adequate CaM level for normal segregation (Davis *et al.*, 1986; Takeda and Yamamoto, 1987; Ohya and Anraku, 1989; Davis, 1992) point out the importance of CaM in the control of chromosome behaviour.

CaM has also been identified as a factor involved in the reinitiation of meiosis in female germ cells. G_2/M transition and maturation phase (Cartaud *et al.*, 1980; Wasserman and Smith, 1981) as well as metaphase/anaphase transition can be triggered by Ca^{2+}-CaM (Lorca *et al.*, 1991, 1993, 1994; Morin *et al.*, 1994). In male germ cells meiosis is not blocked at any stage, appearance and evolution of primary spermatocytes through prophase I is accompained by an increase in both testicular CaM mRNA and CaM content (Lagacé *et al.*, 1981; Feinberg *et al.*, 1983, 1985; D'Agostino *et al.*, 1983; Trejo *et al.*, 1985; Slaughter *et al.*, 1987) and by an enhancement of CaM concentration in the nucleus (Moriya *et al.*, 1993).

The physiological meaning of the rise in CaM level during

meiosis in the male has not been defined. Whether the change in testis CaM content was paralleled by modifications of the targets involved in a specific CaM-mediated calcium response pathway, as previously observed in other cell types (Van Eldik *et al.*, 1990), was pursued in this paper. Subcellular distribution of CaM and CaM-binding proteins (CaMBP) were determined at several stages of testicular development. Rat primary spermatocytes and spermatids were isolated from developing rats as sources of meiotic and postmeiotic CaM targets. Emphasis was made on the identification of nuclear and cytosolic Ca^{2+}-dependent CaMBP in primary spermatocytes at later stages of the first meiotic prophase.

MATERIALS AND METHODS

Minimum essential medium (MEM), without calcium, supplemented with Earle salts was from Gibco, BRL. Collagenase form II was from Koch-Light Laboratories, Ltd. Dextran T-500, CNBr-activivated Sepharose 4B and Ficoll were from Pharmacia Fine Chemicals. Acrylamide, bisacrylamide, Temed, molecular weight protein standards, sodium dodecyl sulfate (SDS), ammonium persulfate, Tris (hydroxymethyl) aminomethane were from BioRad. Phenyl sepharose, sucrose, soy bean trypsin inhibitor (type I) (SBTI), phenylmethylsulfonyl fluoride (PMSF), ethyleneglycol- bis(ß-aminoethyl ether) N,N,N', N',-tetraacetic acid (EGTA), ethylenediamine-tetraacetic acid, tetrasodium salt (EDTA), calcium chloride, bovine serum albumin (BSA), imidazol, boric acid, sodium borate, sodium chloride, dithithreitol (Cleland's Reagent) (DTT), sodium chloride, adenosine-5'-monophosphate (AMP), glucose-6-phosphatase (G6P), sodium succinate, N$_{\alpha}$-CBZ-arg-arg-4-methoxy-ß-naphthylamide, p-nitrophenyl phosphate, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were from Sigma Chemicals, St. Louis Missouri, U.S.A.

Animals. Sprague Dawley male rats were used in all experiments. Following weaning at 21 days, they were fed with Purina chow and water *ad libitum*. Rats were stunned, decapitated and exsanguinated. Testis were removed and received in the apropriate medium. Tunica albuginea and blood vessels were eliminated before cell dispersion or tissue homogenization.

Cell populations. Isolated germ cell populations were obtained

as previously described (Alemán *et al.*, 1978). Primary spermatocytes at leptotene-zygotene or pachytene stages were obtained from 12-15 and from 21-25 day old rat testes, respectively; spermatids were obtained from 38 day old animals. Leptotene-zygotene primary spermatocytes were recovered in the Dextran-MEM layer with a density of 1.0816 (g/ml). Pachytene primary spermatocytes and spermatids in the 1.038 and 1.053 (g/ml) density layers. Cells were resuspended with DPBS (136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.68 mM $CaCl_2$ pH 7.2) to eliminate Dextran T-500. The number of cells obtained was determined in triplicate aliquots, using a Neubauer chamber. Smears of isolated cells were fixed under PBS-3% formaldehyde vapours and stained with PAS-hematoxylin. Cells were identified according to Leblond and Clermont (1952), counting at least 500 cells per experiment. Values shown are the means and standard deviation of three different experiments. At 12-15 days the germ cell population was constituted by 56.25 ± 5.27 % leptotene-zygotene primary spermatocytes, 2.44 ± 0.17 % early pachytene primary spermatocytes, 7.22 ± 0.26 % spermatogonia, 18.81 ± 16.44 % Sertoli cells and by 15.17 ± 10.53 % Leydig cells. At 21 days the primary spermatocyte enriched population consisted of 81.38 ± 1.64 % pachytene primary spermatocytes, 3.14 ± 1.06 % leptotene primary spermatocytes, 7.41 ± 3.02 % spermatogonia, 1.73 ± 0.7 % Sertoli cells and 2.54 ± 0.005 % Leydig cells. The spermatid enriched population obtained from 38 day-old rat testis was integrated by 59.16 ± 8.57 % Golgi phase spermatids, 14.57 ± 5.16 % acrosome and elongating spermatids,

25.42 ± 3.53 % primary spermatocytes, and 2 ± 2.82 % spermatogonia. Spermatozoa were obtained from caput, body and cauda of epididymis. Caput and body epididymal spermatozoa were obtained by mincing these regions in separate flasks containing PBS. The supernatant was filtered through cheese cloth and then centrifuged to 1900 x g 10 min at room temperature. Spermatozoa from the cauda of epididymis were obtained by perfusion with PBS.

Rat testis subcellular fractions. Testicular subcellular fractions were obtained from 13, 21 and 38-day old and adult rats. A 10% (w/v) tissue homogenate was prepared in ice-cold 0.25 mM sucrose, 20 mM Hepes, pH 7.2, using a Potter-Elvejhem homogenizer with a loose teflon pestle. Six subcellular fractions were obtained by differential centrifugation of the homogenate at 770 x g for 10 min (P_1), at 6 000 x g for 20 min (P_2), at 12 500 x g for 40 min (P_3), at 24 000 x g during 40 min (P_4) and at 150 000 x g for 60 min (P_5) and the final supernatant. The activity of several marker enzymes such as succinic dehydrogenase (Ackrell *et al.*, 1978), acid phosphatase (Hollander, 1971), cathepsin B (Smith and Frank, 1975), glucose-6-phosphatase (Schulze *et al.*, 1985), 5'-nucleotidase (Emmelot and Bos, 1966), and of alkaline phosphatase (Perkarthy *et al.*, 1972) were determined to identify each subcellular fraction.

^{125}I-Calmodulin probe. Bovine calmodulin was purified from bovine testes as Gopalakrishna and Anderson (1982) and labeled with ^{125}I using the Bolton-Hunter reagent as described (Chafouleas *et al.*, 1979).

Antibodies. CaM monospecific antibodies were produced in sheep

and purified by affinity chromatography as previously described (Dedman *et al.*, 1978).

CaM content. CaM level was determined by radioimmunoassay (Chafouleas *et al.*, 1979) in the following samples. A) Total homogenate of developing rat testis. Testicular tissue from 5 to 60 day old rats (10 to 2 animals), was homogenized with borate saline (100 mM boric acid, 25 mM sodium borate, 75 mM NaCl, pH 8.4), 2 mM DTT, 1 mM EGTA, 0.1% (v/v) SDS, using a Potter-Elvejhem glass-teflon homogenizer. The homogenate was sonicated for 2 min at 4°C, using a microtip equipped Model B-12 Branson sonicator. Aliquots of 1 ml were heated at 95°C for 2 min in a thermoblock, chilled on ice for 30 min and microcentrifuged for 5 min. Supernatants were saved and frozen at -70°C until CaM content determination. B) Rat testis subcellular fractions. The pellets obtained by differential centrifugation of the testis homogenate were resuspended with borate saline solution plus 1 mM EGTA, 0.1% SDS and 2 mM DTT. The cytosolic fraction was supplemented with SDS and DTT to obtain 0.1% and 2 mM final concentration. Sonication and heat treatments were performed in all fractions as described above. C) Isolated germ cells. Aliquots of 10^6 cells were homogenized with borate saline-EGTA-SDS-DTT and processed as described for tissue and subcellular fractions.

Electrophoresis. Aliquots, 80 μg of protein, of testis homogenate, isolated primary spermatocytes and their subcellular fractions were fractionated by SDS-polyacrylamide slab gel electrophoresis as described by Laemmli (1970). Electrophoresis was

run overnight at 25 volts.

Identification of Ca^{2+}-dependent and Ca^{2+}-independent CaMBP. The identification of Ca^{2+}-dependent and Ca^{2+}-independent CaMBP was made by ^{125}I-CaM overlay in the presence of 2 mM $CaCl_2$ or with 4 mM EGTA (Glenney and Weber, 1980) in the following samples: A) Testicular tissue. Rat testis, free from tunica albuginea and blood vessels, obtained from 10, 20 and 40 day old animals, were homogenized with sample buffer previously heated to 95 °C, to obtain a 10% (w/v) homogenate and equivalent protein aliquots were separated in 10% polyacrylamide gels. B) Subcellular fractions. C) Isolated primary spermatocytes and spermatids. D) Purified nuclear and cytoplasmic fractions from primary spermatocytes.

Nuclei purification from isolated primary spermatocytes. Pachytene primary spermatocyte nuclei were obtained following the techinque described by Birnie (1978) with some modifications. Briefly, isolated cells were homogenized with ice-cold 0.25 mM sucrose, 20 mM imidazol pH 7.2, 1 mM EDTA, 100 μg/ml SBTI, using a Potter Elvejhem glass homogenizer with a loose teflon pestle. Cellular homogenate was centrifuged at 800 x g 10 min at 4°C. The supernatant was saved and the pellet was rehomogenized and centrifuged as described above. The pellet was resuspended with 4 ml of chilled 20 mM imidazol, 4 mM EDTA, 100 μg/ml SBTI, pH 7.2. After 10 min the suspension was layered on top of 0.88 M sucrose, 20 mM imidazol, 1 mM EDTA, 100 μg/ml SBTI, pH 7.2 and centrifuged at 800 x g 10 min at 4°C. The nuclear pellet was resuspended with 0.25 mM sucrose, 5 mM $MgSO_4$, 1 mM PMSF, 50 mM Tris-HCl pH 7.4,

aliquoted and kept at -80°C. To define the contamination of the nuclear fraction with cytosolic and endoplasmic reticulum proteins, the activities of lactate dehydrogenase (LDH) (Machado de Domenech *et al.*, 1972) and glucose-6-phosphatase (Schulze *et al.*, 1985) were determined in the cell homogenate, in the supernatant obtained after centrifugation at 800 x g, and in the pellet of nuclei obtained after centrifugation on sucrose 0.88 M.

Protein determination. Protein was determined by the Hartree (1972) or Bradford (1976) method.

RESULTS

Testis growth and CaM content. Previous observations showed that CaM gradually increased during testis development (Smoake *et al.*, 1974; Lagacé *et al.*, 1981; Feinberg *et al.*, 1983; Trejo *et al.*, 1985; Slaughter *et al.*, 1987). However, the relationship between testis growth rate and CaM content was not defined. To accomplish this, individual testis weight, total protein as well as CaM content per g of tissue were determined in developing rats from 5 to 60-days old, at 2 day intervals. Fig. 1 shows that testis weight increases exponentially from 5 up to 40 days but the protein content per g of wet tissue was constant from birth to adulthood. The water content per g of tissue did not change (not shown). The CaM:total protein ratio increased in a step-manner along the logaritmic growth phase. The first rise was observed between 5-10 days after birth and the level reached was maintained up to 20 days. This period of testicular development is characterized by the appearance and proliferation of spermatogonia and Sertoli cells (Clermont and Perey, 1957). Sertoli cell number per testis increases 4 fold between 5-10 days and spermatogonia proliferation peaks between 10 and 20 days. Primary spermatocytes are clearly observed in the 15-day old rat testis (Zhengwei *et al.*, 1990). The second increase in testicular CaM content was detected in 24 day old rats and this level was mantained up to 32 days. During this interval of time the last stages of meiosis are reached and haploid cells appear in the germinal epithelium (Clermont and Perey, 1957). At 25 days primary spermatocytes are the predominat cell type in

the rat germinal epithelium (Zhengwei *et al.*, 1990). The third rise in tetis CaM was observed at 34 days and was constant up to 40 days. Within this phase of testis development, spermatid cell number increases, acrosome and cap phases of spermiogenesis are attained (Clermont and Perey, 1957). Spermatogonia and primary spermatocytes reach the cell density that will be preserved in the adult germinal epithelium (Zhengwei *et al.*, 1990). Plateau phase of growth, spermatid maturation and spermatozoa appearance in the germinal epithelium at 45 days, were coincident with decreasing level of testicular CaM. Whether the elimination of cytoplasmic components during last stages of spermatid maturation as well as spermatid degeneration (Clermont and Perey, 1957) cause the decrease in CaM between 40-45 days remains to be defined.

CaM content in isolated germ cells. Spermatogenic cells at specific stages of differentiation were isolated to determine their CaM content on cell and protein basis. The earliest meiotic cells, leptotene and zygotene primary spermatocytes isolated from 15 day old rats, showed a CaM level 10 times higher than the content determined in spermatogonia and Sertoli cells isolated from 12 day old rats. Pachytene primary spermatocytes, from 21 day old rats, showed a 2 fold higher CaM content than early spermatocytes. A decrease in CaM level was observed in postmeiotic cells, spermatids isolated from 38 day old rats, showed 20% less CaM and epididymal spermatozoa from adult rats, presented eight times lower CaM content than pachytene primary spermatocytes (Fig. 2). These differences were also observed when CaM level was expressed as

percentage of germ cell protein. CaM content increased from 0.048% in spermatogonia and Sertoli cells to 0.23% in early primary spermatocytes and to 0.31% in pachytene primary spermatocytes. In spermatids the total protein content decreased and CaM level attained the 0.45%. The present results support and extend previous reports in which CaM content was estimated on cell basis by stimulation of Ca^{2+}-calmodulin dependent enzymes using germ cell extracts (D'Agostini *et al.*, 1983; Feinberg *et al.*, 1983).

Ca^{2+}-dependent CaMBP in developing rat testis. Rat testis at basal, logaritmic and plateau stages of the growth curve were selected to define whether CaM content was related with changes in CaM targets. Ca^{2+}-dependent CaMBP were detected in rat testis homogenates at 5, 20 and 40 days by ^{125}I-CaM overlay (Glenney and Weber, 1980). In spite of the differences in CaM level, a similar pattern of Ca^{2+}-CaMBP was observed at the three stages of testis development (Fig. 3). However, in the logaritmic phase of growth, the 20 day-old rat testis showed a higher content in the proteins with apparent M_r = 140, 60, 47, 23-22, 21, 18, 17 and 14 kD. A 27 kD protein was detected only at 20 and 40-day old rat testis. At 20 days, primary spermatocytes and spermatogonia are the major cell types in the germinal epithelium, the primary spermatocytes:spermatogonia ratio is 0.59 (Zhengwei *et al.*, 1990). At 40 days, both the plateau stage of growth and the highest testicular CaM level were reached. At this age spermatids are the predominant cell type, the spermatid:primary spermatocyte ratio is 1.65 and the primary spermatocyte:spermatogonia ratio is 2.38

(Zhengwei *et al.*, 1990). The results obtained suggest that the changes in the level of Ca^{2+}-dependent CaMBP are related to the growth rate and to the cell types present in the germinal epithelium rather than to the total CaM content.

Subcellular distribution of CaM and CaMBP. To define whether the changes in testis CaM content could be related to modifications in its cellular localization and/or in the level of CaMBP, subcellular fractions from immature and adult rat testis were obtained and assayed for both CaM and CaMBP. 80% of the total CaM content of the developing and mature testis was recovered in P_1 and in the final supernatant. However, in the testis from adult rats, 50 % of the total CaM was recovered in P_1 and in immature rat testis this percentage was recovered in the soluble fraction. At both stages of development, 10% of the total CaM content was recovered in P_2 (6 000 x g), the mitochondrial-lysosomal fraction. A 2-3% of CaM was recovered in each one of the vesicles derived from the endoplasmic reticulum and plasma membrane sedimented in P_3, P_4 and P_5 (Fig. 4).

The subcellular localization of the Ca^{2+}-dependent CaMBP was studied at 13, 20, 37-days and adult rat testis. Proteins were separated in 6.5% polyacrylamide gels to better visualize the high molecular weight proteins and in 13% polyacrylamide gels to resolve the dense label observed in the region of low molecular weight. *Electrophorus electricus* CaMBP purified by affinity chromatography were included as internal control to asses the identification of the testis CaMBP (Kaetzel and Dedman, 1987). The distribution

profile of Ca^{2+}-CaMBP was similar at all the ages studied, however a higher content was observed in 13 to 20 day old rat testis, when meiotic cells are the major cell type in the germinal epithelium. High molecular weight Ca^{2+}-CaMBP with an apparent M_r = 260, 210, 170 and 150 kD, were mainly localized in the cytosol and in the membrane vesicles pelleted at 24 000 (P_4) and 150 000 x g (P_5) derived from endoplasmic reticulum and plasma membrane (Fig. 5A). P_4 and P_5 were also enriched in a 35 and 27 kD proteins (Fig. 5B). A 60 kD Ca^{2+}-dependent CaMBP was detected in all fractions but seemed to predominante in the cytosol (Fig. 5A). Two proteins with an apparent molecular weight of 17 and 13 kD were only observed in P_1 (Fig. 5B). These results suggested that CaM had multiple targets, those with high molecular weight seemed to be mainly localized in the cytosol and those with a low molecular weight localized in the nuclear and light membrane fractions P_4 and P_5.

Ca^{2+}-dependent CaMBP in isolated primary spermatocytes and spermatids. In order to define whether meiotic and postmeiotic cells show specific CaMBP, pachytene primary spermatocytes and spermatids were isolated and their Ca^{2+}-CaMBPs were identified (Fig. 6). Similar high and low molecular weight Ca^{2+}-dependent CaM-acceptor proteins were detected in both cell types, M_r = 220, 180, 140, 120, 100, 90, 74, 55, 43, 40, 33, 17, 16 and 13 kD. Although, a more dense label was observed in some CaMBP in primary spermatocytes (Fig. 6). These results stand in contrast to the qualitative and quantitative differences between the Ca^{2+}-CaMBP detected in isolated germ cells from 12 day old rat testis,

enriched in spermatogonia plus Sertoli cells, and those CaMBP shown in pachytene primary spermatocytes (Fig. 6 lanes 1,2).

CaMBP in purified nuclei from primary spermatocytes. Since CaM binds to the nucleus at later stages of the first meiotic prophase, isolated primary spermatocytes at pachytene and later stages were selected to identify the nuclear CaMBP. Several Ca^{2+}-dependent CaMBP were detected in the purified primary spermatocyte nuclear fraction M_r= 200, 120, 100, 82, 74, 56, 52, 43, 37, 35, 32 and 29 kD. The 56 kD band was predominant in the cytoplasm and in the purified nuclei preparation. A group of proteins with low molecular weight 37-31 kD seemed to be only present in the nucleus (Fig. 7). The molecular weight of some of these CaMBP resemble the proteins able to bind CaM detected in the cytosol obtained from immature rat testis (Fig.5). The possibility that these proteins represent a contamination with cytosolic proteins seems unlikely, since the percentage of lactate dehydrogenase activity recovered in this fraction was 2.93 ± 5 (n=3). However, 43 ± 3 % of glucose-6-phosphatase activity was recovered in this fraction, indicating a high contamination with endoplamsic reticulum proteins. This explanation is supported by the similar molecular weight CaMBP detected in the membrane vesicles pelleted at 24 000 (P_4) and 150 000 x g (P_5) (Fig. 5).

The presence of Ca^{2+}-independent CaMBP of low molecular weight was exclusively detected in the nuclear fraction obtained from primary spermatocytes (Fig. 7).

DISCUSSION

The specific increase in CaM content expressed on CaM/protein basis observed at three specific stages of testicular development confirmed and extend previous observations (Smoake *et al.*, 1974; Lagacé *et al.*, 1981; Feinberg *et al.*, 1983, 1985; Slaughter *et al.*, 1987). However, these results stand in contrast to a transient increase of CaM observed in cells induced to proliferate (MacManus *et al.*, 1981; Piñol *et al.*, 1988), and with cycling cells which double their CaM content at the G_1/S transition (Chafouleas *et al.*, 1982), but maintain a constant CaM/total protein ratio (reviewed by Veigl et al., 1984). The first rise in CaM, between 5-10 days, takes place during spermatogonia and Sertoli cell proliferation (Clermont and Perey, 1952). The second and third rises in testis CaM may be related to spermatogonia renewal and appearance of new generations of primary spermatocytes. The increase in primary spermatocyte number up to 35 days supports this interpretation (Zhengwei *et al.*, 1990). Synthesis of CaM during meiosis appears to be a common feature of both male and female meiotic cells. However, in *Xenopus* oocytes CaM level only increased 70% during germinal vesicle breakdown (Cartaud *et al.*, 1980). The relationship between CaM content decrease observed between 20-22 days and 40-45 days and germ cell degeneration remains to be defined.

The relevance of primary spermatocytes at pachytene stage as the cell type in which specific testis gene expression takes place is supported by numerous reports (reviewed by Eddy *et al.*, 1993; Hecht, 1993). The higher Ca^{2+}-dependent CaMBP level detected at 20

days, when germ cell differentiation have reached the pachytene stage further support the importance of this meiotic stage. However, a similar Ca^{2+}-CaMBP pattern was observed in both isolated primary spermatocytes and spermatids, some bands showed a stronger label in meiotic cells. These results may indicate common CaM targets in both cell types, although the role they play remains to be defined. It seems likely that the 33 kD Ca^{2+}-dependent CaMBP detected in the spermatid-enriched population correspond to calspermin, the specific Ca^{2+}-CaMBP of the testis expressed in postmeiotic cells (Ono *et al.*, 1984, 1987, 1989).

Cytosol appeared to be one of the major subcellular compartments where CaM and Ca^{2+}-CaMBP are distributed in rat testis at different developmental stages. Testis cytosol was enriched in high molecular weight Ca^{2+}-CaMBP that resemble the subunits of cytoplasmic dynein, a major microtubule-associated protein in tat testis (Neely *et al.*, 1990). It seems very likely that the 400 kD and the 80 kD Ca^{2+}-CaMBP detected by Sobue and Kakiuchi (1980) in adult rat testis cytosol correspond to cytoplasmic dynein subunits. Although changes in isotypes and organization of the major cytoskeletal constituents have been observed during spermatogenesis in both male germ cells and Sertoli cells (Lewis and Cowan, 1988; Kim *et al.*, 1989; Vogl, 1989), the role of CaM in the regulation of this process has not been defined.

The possibility that some of the Ca^{2+}-dependent CaMBP detected in the purified nuclei were related to nuclear cytoskeleton is very likely. The contamination with soluble proteins and therefore with

cytoplasmic cytoskeleton was very low or undetectable by enzymatic methods. Furthermore, myosin and actin have been immunolocalized in the nuclei of primary spermatocytes (Martino *et al.*, 1980), and changes in myosin ligh chain kinase, -spectrin, actin and caldesmon have been detected in the nuclei of proliferatively activated rat liver cells (Bachs *et al.*, 1990). The contamination of the purified nuclei with endoplasmic reticulum proteins makes difficult to define whether the nuclear membrane, still assembled in primary spermatocytes, and endoplasmic reticulum show common Ca^{2+}-CaMBP.

The nuclear compartment of isolated pachytene primary spermatocytes also showed proteins able to bind CaM in the absence of Ca^{2+}. A similar result was not observed in any other subcellular fraction. Ca^{2+}-dependent, Ca^{2+}-independent and Ca^{2+}-inhibited CaMBP have been reported in different cell types (Glenney and Weber, 1980; Cimier *et al*, 1985; Andreasen *et al*, 1987; Hernández *et al.*, 1994), but not in dividing cells. The present resuslts support previous observations suggesting the existence of Ca^{2+}-independent CaMBP in dividing cells since Ca^{2+}-chelating agents did not affect CaM localization in the kinetochore microtubules (Sweet *et al.*, 1988). Whether the low molecular weight CaMBP able to bind CaM in the presence of EGTA could be involved in the association of CaM to nuclear structures remains to be defined.

The Ca^{2+}-dependent binding of CaM to the particulate fraction of adult rat testis was shown by Sobue *et al* (1979), but the binding proteins were not identified. This report is the first

showing the subcellular distribution of CaM, CaMBP and their relation with the growth rate and differentiation stage attained by the germinal epithelium. Studies are in progress to stablish the identity and functions mediated by the CaM-CaMBP system in primary spermatocytes.

CONCLUSIONS

Testis growth rate, meiosis progresion and differentiation of spermatids were associated with an increase in CaM:total protein ratio. CaM content rised at three specific stages of development and the changes were not transient. CaM
was mainly compartmentalized in the nuclear and cytosol fractions during the exponential and plateau phases of testis growth. Several Ca^{2+}-CaMBP were detected in rat testis with higher level during the exponential growth phase. Primary spermatocytes and early spermatids showed a similar pattern of Ca^{2+}-dependent CaMBP, suggesting common CaM targets in meiotic and early postmeiotic cells. Ca^{2+}-independent CaMBP were detected in primary spermatocytes nuclei. The role played by both Ca^{2+}-dependent and Ca^{2+}-independent CaMBP remains to be defined.

BIBLIOGRAFPHY

Ackrell BAC, Kearney EB, Singer TP (1978): Mammalian succinate dehydrogenase. In Fleischer S, Packer L (eds): "Methods in Enzymology". Part D Biological Oxidations. Mitochondrial and Microbial Systems. Vol. LIII. Academic Press, New York. pp 466-483.

Alemán V, Trejo R, Morales E, Hernández-Jáuregui P, Delhumeau-Ongay G (1978): A simple and rapid technique to isolate enriched populations of spermatocytes and spermatids from the immature rat testis. J Reprod Fert 56:67-75.

Andreasen TJ, Luetje CW, Heideman W, Storm DR (1983): Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. Biochem 22: 4615-4618.

Bachs O, Lanini L, Serratosa J, Coli MJ, Basts R, Aligué R, Rius E, Carafoli E (1990): Calmodulin-binding proteins in the nuclei of quiescent and proliferatively activated rat liver cells. J Biol Chem 265:18595-18600.

Birnie GD (1978): Isolation of nuclei from animal cells in culture. In Stein G, Stein J, Kleinsmith LJ (eds): "Methods in Cell Biology". Vol. XVII. Chromatin and Chromosomal Protein Research II. Academic Press, New York. pp. 13-26.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein using the

principle of protein-dye binding. Analyt Biochem 72:248-254.

Cartaud A, Ozon R, Walsh MP, Haeich J, Demaille JG (1980): *Xenopus laevis* oocyte calmodulin in the process of meiotic maturation. J Biol Chem 255: 9404-9408.

Chafouleas JG, Dedman JR, Munjaal RP, Means AR (1979): Calmodulin. Development and application of a sensitive radioimmunoassay. J Biol Chem 254:10262-10267.

Chafouleas JG, Bolton WE, Hidaka H, Boyd AE III, Means AR (1982): Calmodulin and the cell cycle:involvement in regulation of cell cycle progression. Cell 28:41-50.

Cimier B, Andreasen TJ, Andreasen KI, Stormn DR (1985): P-57 is a neural specific calmodulin-binding protein. J Biol Chem 260:19784-10788.

Clermont Y, Perey B (1957): Quantitative study of the cell population of the seminiferous tubules in immature rats. Amer J Anat 100:241-267.

D'Agostino A, Monaco L, Conti M, Geremia R (1983): Calmodulin in mouse male germ cells: a qualitative and quantitative study. Cell Differ. 13:35-40.

Davis TN, Urdea MS, Masiarz FR, Thorner J (1986): Isolation of the yeast calmodulin gene: Calmodulin is an essential protein. Cell 47:423-431.

Davis TN (1992): A temperature-sensitive calmodulin mutant loses viability during mitosis. J Cell Biol 118:607-

617.

Dedman JR, Welsh MJ, Means AR (1978): Ca^{2+}-dependent regulator: Production and characterization of monospecific antibody. J Biol Chem 253:7515-7521.

DeMartino C, Capanna E, Nicotra MR, Natali PG (1980): Immunochemical localization of contractile proteins in mammalian meiotic chromosomes. Cell Tissue Res 213:159-178.

Eddy, EM, Welch JE, O'Brien DA (1993): Gene expression during spermatogenesis. In de Kretser D (ed): "Molecular Biology of the Male Reproductive System". Academic Press, San Diego, CA. pp 181-232.

Emmelot P, Boss CJ (1966): Studies on plasma membrane III. Mg^{2+}-ATPase, (Na^{+}-K^{+}-Mg^{2+})-ATPase and 5'nucleotidase activity of plasma membrane isolated from rat liver. Biochim Biophys Acta 210:369-382.

Feinberg J, Pariset C, Rondard M, Loir M, Lanneau M, Weinman S, Demaille J (1983): Evolution of Ca^{2+}- and cAMP-dependent regulatory mechanisms during ram spermatogenesis. Dev Biol 100:260-265.

Feinberg J, Pariset C, Weinman S (1985): Calmodulin level and cAMP-dependent protein kinase activity in rat spermatogenic cells and hormonal control of spermatogenesis. Dev Biol 108:179-184.

Geiser RJ, van Tuinen D, Brockerhoff SE, Neff MM, Davis TN (1991): Can calmodulin function without binding calcium?.

Cell 65:949-959.

Glenney JR Jr, Weber K (1980): Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. J Biol Chem 255:10551-10554.

Gopalakrishna R, Anderson WB (1982): Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem Biophys Res Commun 104:830-836.

Hartree EF (1973): Determination of protein. A modification of the Lowry method that gives a linear photometric response. Analyt Biochem 48:422-426.

Hecht NB (1993): Gene expression during male germ cell development. In Desjardins D, Ewing LL (eds): "Cell and Molecular Biology of the Testis". Oxford University Press, New York, NY. pp 400-432.

Hernández EO, Trejo R, Espinoza AM, González A, Mújica A (1994): Calmodulin binding proteins in the membrane vesicles released during the acrosome reaction and in the perinuclear material in isolated acrosome reacted sperm heads. Tissue Cell 26:849-865.

Hollander VP (1971): Acid phosphatases. In Boyer PD (ed): "The Enzymes" Vol. IV. Chap. 19. Academic Press New York and London. pp 449-498.

Kaetzel MA, Dedman JR (1987): Identification of a 55 kDa high affinity calmodulin-binding protein from *Electrophorus*

electricus. J Biol Chem 262:1818-1822.

Kim E, Waters SH, Hake LE, Hecht NB (1989): Identification and developmental ecpression of a smooth-muscle γ-actin in postmeiotic male germ cells of mice. Mol Cell Biol 9:1875-1881.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lagacè L, Chafouleas G, Trejo R, Delhumeau-Ongay G, Dedman JR (1981): Calmodulin levels during rat testis development and intracellular localization in intact cells. Biol Reprod 22 (Suppl.1):53A.

Leblond CP, Clermont Y (1952): Spermatogenesis of rat, mouse, hamster and guinea-pig as revealed by the periodic acid-fuchsin sulfurous acid technique. Am J Anat 90:167-215.

Lewis SA, Cowan NJ (1988): Complex regulation and functional versatility of mammalian α- and ß-tubulin isotypes during the differentiation of testis and muscle cells. J Cell Biol 106:2023-2033.

Lorca T, Galas S, Fesquet D, Devault A, Cavadore JC, Dorée M (1991): Degradation of the proto-oncogene product $p39^{mos}$ is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca^{2+}-calmodulin dependent event. EMBO J 10:2087-2093.

Lorca T, Cruzalegui FH, Fesquet D, Cavadore JC, Means A, Dorée M (1993): Calmodulin-dependent protein kinase II mediates

inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. Nature 366:270-273.

Lorca T, Abrieu A, Means A, Dorée M (1994): Ca^{2+} is involved through type II calmodulin-dependent protein kinase in cyclin degradation and exit from metaphase. Biochim Biophys Acta 1223:325-332.

Machado de Domenech E, Domenech CE, Aoki A, Blanco A (1972): Association of the testicular lactate dehydrogenase isozyme with special type of mitochondria. Biol Reprod 6: 136-147.

MacManus JP, Braceland RB, Rixon RH, Whitfield JF, Morris HP (1981): An increase in calmodulin during growth of normal and cancerous liver in vivo. FEBS Lett:133:99-102.

Marcum JM, Dedman JR, Brinkley BR, Means AR (1978): Control of microtubule assembly-disassembly by calcium-dependent regulator protein. Proc Natl Acad Sci USA 75:3771-3775.

Mills NC, Means AR (1972): Sorbitol dehydrogenase of rat testis: Changes of activity during development, after hypophisectomy and following gonadotropic hormone adminstration. Endocrinol 91:147-156.

Morin N, Abrieu A, Lorca T, Martin F, Dorée M (1994): The proteolysis-dependent metaphase to anaphase transition: calcium/calmodulin-dependent protein kinase II mediates the onset of anaphase in extracts prepared from unfertilized *Xenopus* eggs. EMBO J 13:4343-4352.

Moriya M, Katagiri C, Yagi K (1993): Immuno-electron

microscopic localization of calmodulin and calmodulin-binding proteins in the mouse germ cells during spermatogenesis and maturation. Cell Tissue Res 271:441-451.

Neely MD, Erickson HP, Boekelheide K (1990): HMW-2, the Sertoli cell cytoplasmic dynein from rat testis, is a dimer composed of nearly identical subunits. J Biol Chem 265:8691-8698.

Ohya Y, Anraku Y (1989): A galactose-dependent *cmd1* mutant of *Saccharomyces cerevisiae*: involvement of calmodulin in nuclear division. Curr Genet 15:113-120.

Ono T, Koide Y, Arai Y, Yamashita K (1984): Heat-stable calmodulin-binding protein in rat testis. Inhibition of calmodulin-stimulated cyclic nucleotide phosphodiesterase activity. J Biol Chem 259:9011-9016.

Ono T, Koide Y, Arai Y, Yamashita K (1987): Tissue specificty of calspermin: A heat-stable M_r 32,000 calmodulin-binding protein. Arch Biochem Biophys 255:102-108.

Ono T, Slaughter GR, Cook RG, Means AR (1989): Molecular cloning sequence and distribution of rat calspermin, a high affinity calmodulin-binding protein. J Biol Chem 264:2081-2087.

Pekarthy JM, Short J, Lansing AI, Lieberman I (1972): Function and control of liver alkaline phosphatase. J Biol Chem 247:1767-1744.

Piñol MR, Berchtold MW, Bachs O, Heizmann C (1988): Increased

calmodulin synthesis in the pre-replicative phase of rat liver regeneration. FEBS Lett 231:445-450.

Rasmussen CD, Means AR (1987): Calmodulin is involved in regulation of cell proliferation. EMBO J 6:3961-3968.

Rasmussen CD, Means AR (1989): Calmodulin is required for cell-cycle progression during G_1 and mitosis. EMBO J 8:73-82.

Schulze HU, Kannler R, Junker B (1985): Latency studies on rat liver microsomal glucose-6-phosphatase. Correlation of membrane modification and solubilization by Triton X-114 with the enzymatic activity. Biochim Biophys Acta 814:85-95.

Slaughter GR, Needlman DS, Means AR (1987): Developmental regulation of calmodulin, actin, and tubulin RNAs during rat testis differentiation. Biol Reprod 37:1259-1270.

Smith RE, Frank RM (1975): The use of aminoacid derivates of 4-methoxy-ß-naphtylamine for the rat assays and subcellular localization of tissue proteinases. In Dingle RT, Dean RT (eds): "Lysosomes in Biology and Pathology". Vol. 4. North Holland Press, New York. pp 193-249.

Smoake JA, Song SY, Cheung WY (1974): Cyclic 3′, 5′-nucleotide phosphodiesterase, Distribution and developmental changes of the enzyme and its protein activator in mammalian tissues and cells. Biochem Biophys Acta 341:402-411.

Sobue, K, Muramoto Y, Yamazaki R, Kakiuchi S (1979): Distribution in rat tissues of modulator-binding protein of particulate nature. FEBS Lett 105:105-109.

Sobue K, Kakiuchi S (1980): Modulator binding proteins of testis. Biochem Biophys Res Commun 93:850-856.

Takeda T, Yamamoto M (1987): Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. Proc Natl Acad Sci USA 84:3580-3584.

Trejo R, Turner A, Dedman JR (1985): Changes in calmodulin mRNA during rat spermatogenesis. J Cell Biol 101:369a.

Van Eldik LJ, Zimmer WE, Barger SW, Watterson DM (1990): Perturbation of the calmodulin system in transformed cells. In Pochet R, Lawson EM, Heizmann CW (eds) "Calcium Binding Proteins in Normal and Transformed Cells". Plenum Press, New York. pp 111-120.

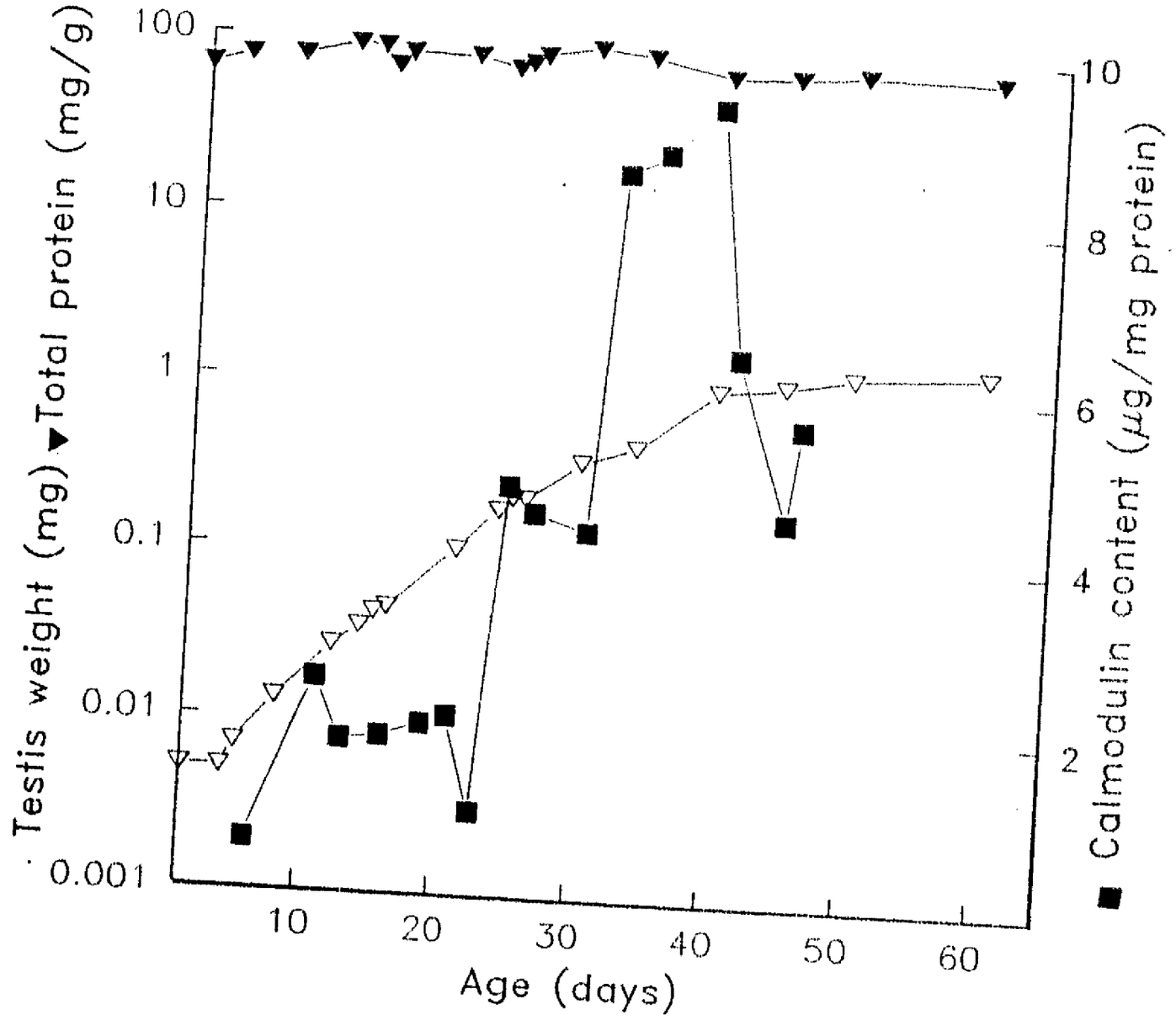
Veigl ML, Vanaman TC, Sedwick WD (1984): Calcium and calmodulin in cell growth and transformation. Biochim. Biophys Acta 738:21-48.

Vogl AW (1989): Distribution and function of organized concentrations of actin filaments in mammalian spermatogenic cells and Sertoli cells. Int Rev Cytol 119:1-56

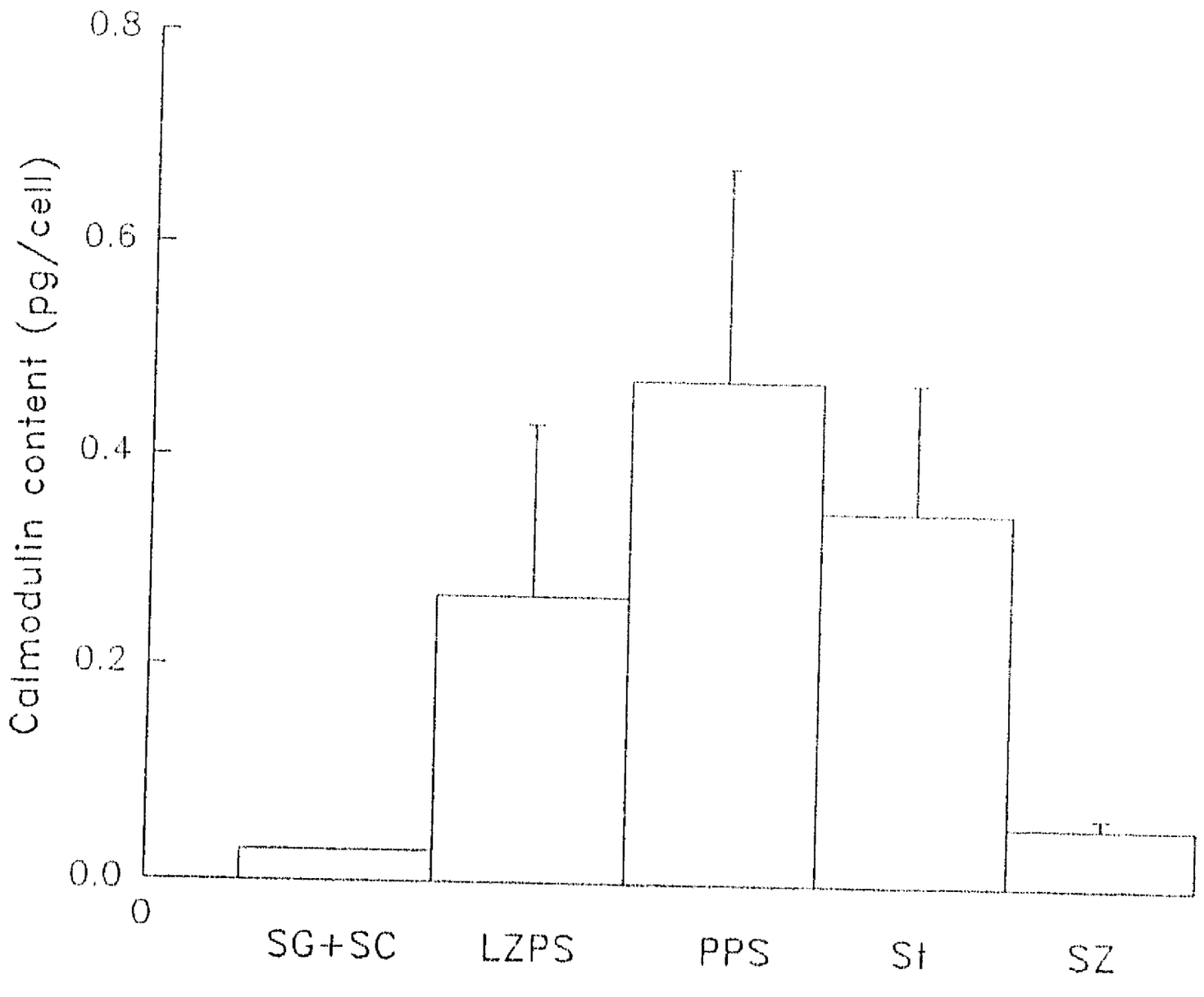
Wasserman WJ, Smith LD (1981): Calmodulin triggers the resumption if meiosis in amphibian oocyte. J Cell Biol 89:389-394.

Welsh MJ, Dedman JR, Brinkley BR, Means AR (1978): Calcium-dependent regulator protein: Localization in mitotic apparatus of eukaryotic cells. Proc Natl Acad Sci USA 75: 1867-1871.

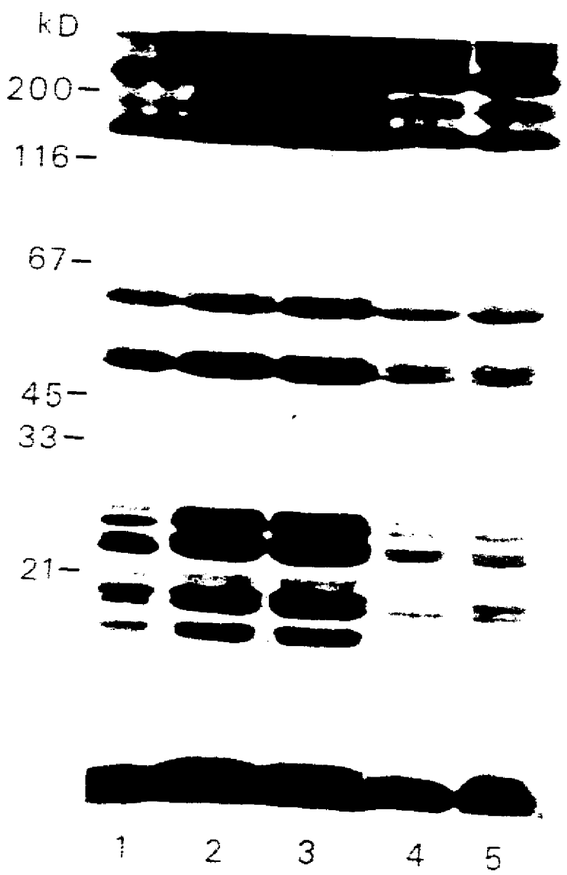
Zhengwei Y, Wreford NG, de Kretser DM (1990): A quantitative study of spermatogenesis in the developing rat testis. Biol Reprod 43:629-635.


Testis weight (mg) ▼Total protein (mg/g)
100
10
1
0.1
0.01
0.001
10
20
30
40
50
60
Age (days)
10
8
6
4
2
■ Calmodulin content (μg/mg protein)

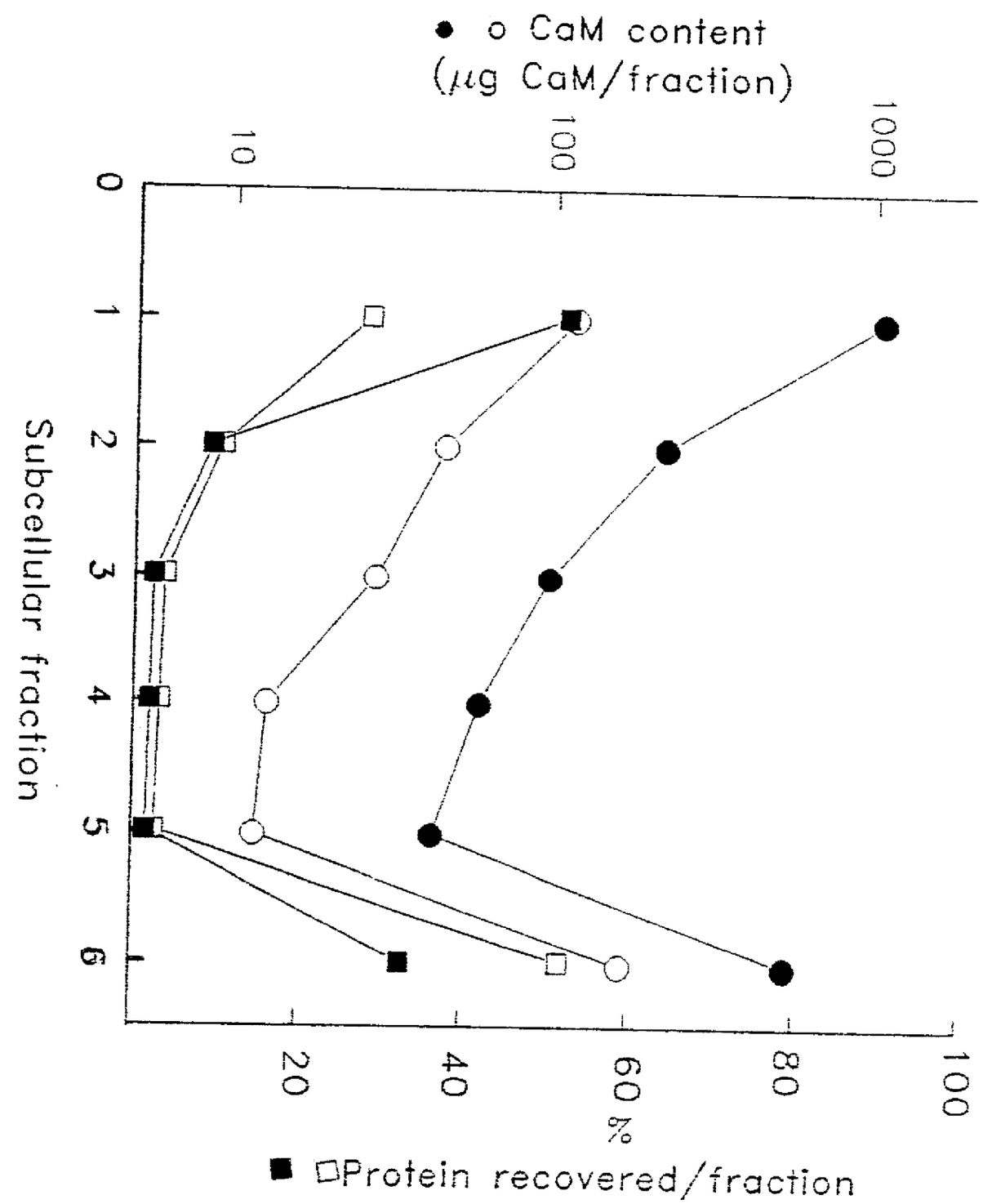
Text. Fig. 1. Correlation between CaM:total protein ratio, testis growth and germinal epithelium differentiation in developing rats. Testis from 5 to 60 day old rats were homogenized in borate saline solution pH 8.4 supplemented with 2 mM DTT, 1 mM EGTA, 0.1% SDS and sonicated for 2 min at 4°C. The homogenate was heated at 95°C for 2 min, chilled on ice for 30 min and microcentrifuged. CaM content was determined by radioimmunoassay in triplicate aliquots of the supernatant. Heated and unheated standards were used for calibration curves (Chafouleas et al., 1979). Testis weight values were obtained from pooled tissue from 50 to 10 male rats. Protein content was determined by the Hartree method (1972).


0.8
0.6
0.4
0.2
0.0
0
Calmodulin content (pg/cell)
SG+SC
LZPS
PPS
St
SZ

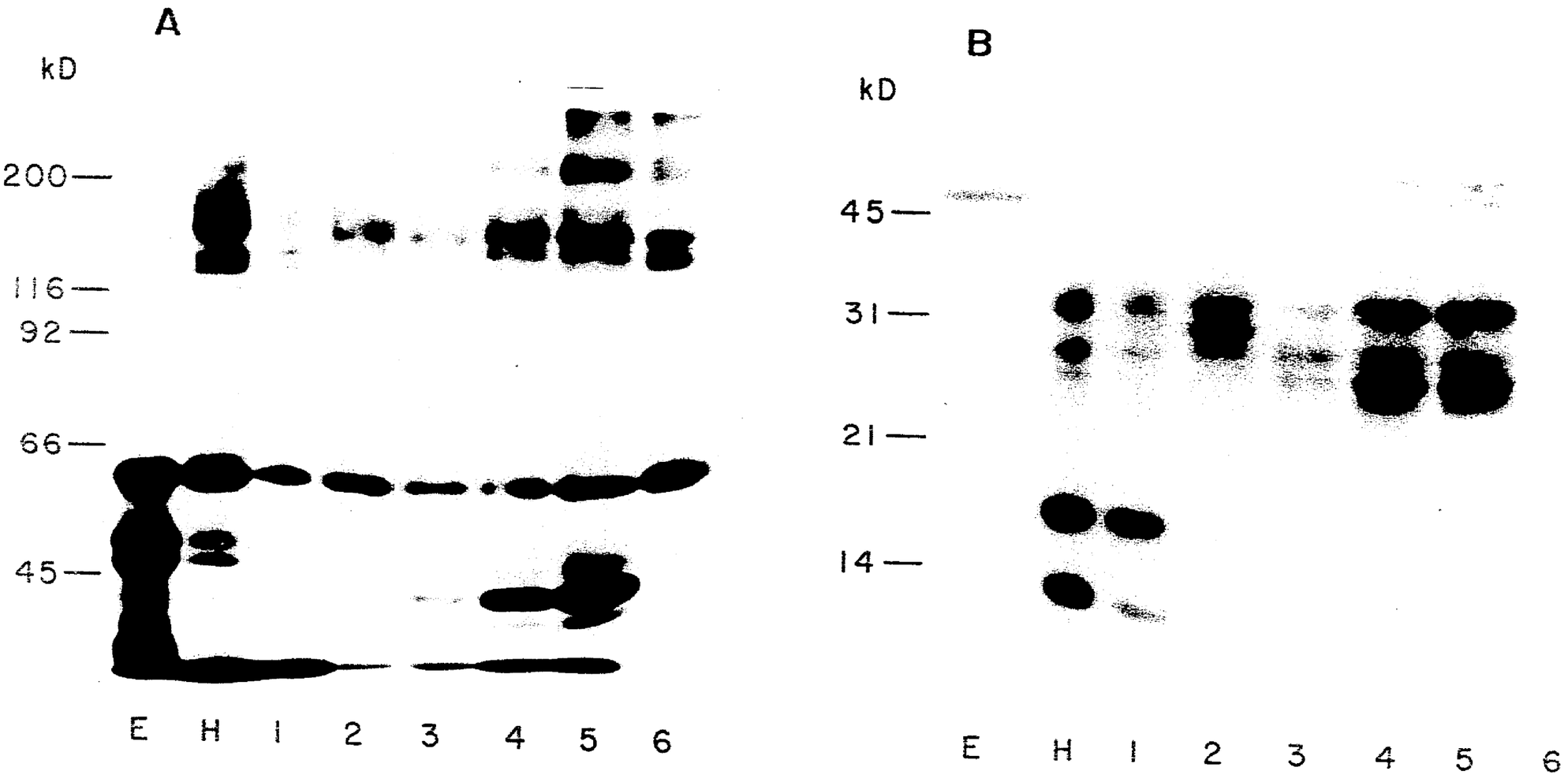
Text Fig. 2. Calmodulin content in isolated germinal cells. Spermatogenic cells were purified from rat testis as described (Alemán et al., 1978). Spermatogonia plus Sertoli cell population was obtained by collagenase and mechanic disruption of testis from 12 day old rats. Leptotene-zygotene primary spermatocytes, pachytene primary spermatocytes and spermatids were isolated from 12-15, 21 and 38 day old rats, respectively, using Dextran T-500-MEM gradients. Spermatozoa were obtained from adult rat epidydimis. Cells were homogenized with borate saline pH 8.4, containing 2 mM DTT, 1 mM EGTA, 0.1% SDS, sonicated and heated as described in Materials and Methods. CaM content was determined by radioimmunoassay in triplicate aliquots. Data shown are the means and standard deviation from 4 different experiments. (SG+SC) spermatogonia and Sertoli cells. (LZPS) leptotene and zygotene primary spermatocytes. (PPS) pachytene primary spermatocytes. (St) spermatids. (SZ) epididymal spermatozoa.


kD
200–
116–
67–
45–
33–
21–
1
2
3
4
5

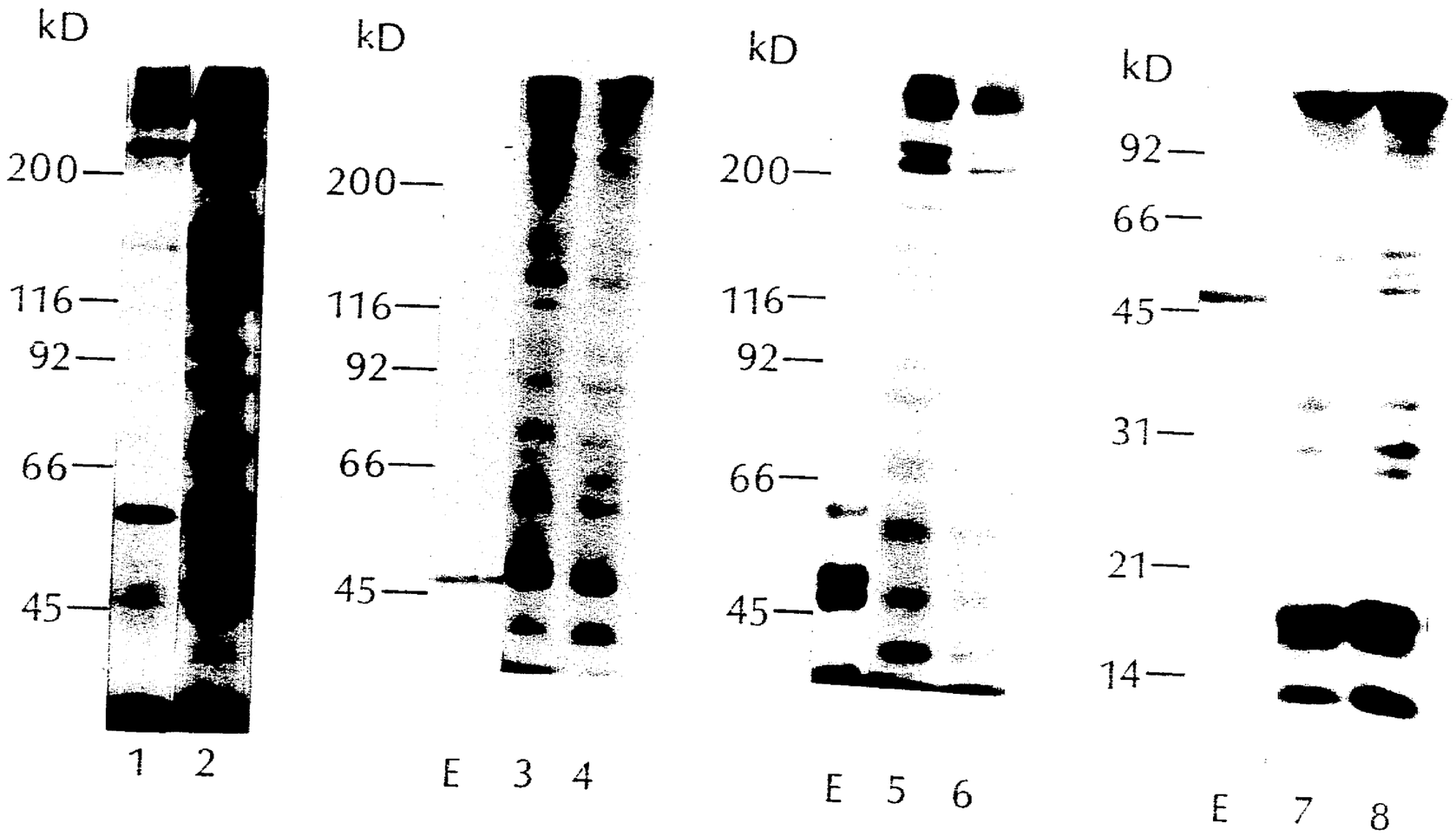
Text Fig. 3. Ca^{2+}-CaMBP during rat testis development. Testicular tissue from 5, 20 and 40 day old rats was homogenized with sample buffer previously heated at 95°C. Aliquots of 80 μg of protein were separated in 10% PAGE-SDS. Gels were overlaid with ^{125}I-CaM, 10^6 dpm per lane, in the presence of 2 mM $CaCl_2$ or 4 mM EGTA. After overnight incubation at 4°C, non bound CaM was eliminated by extensive washing with buffer containing $CaCl_2$ or EGTA. CaMBP were detected on Kodak film by autoradiography. Lane (1), testis from 5 day old rats.Lanes (2) and (3), 20 day old rat testis. Lanes (4) and (5) 40 day old rat testis.


● o CaM content
(μg CaM/fraction)
10
100
1000
0
1
2
3
4
5
6
Subcellular fraction
20
40
60
80
100
%
■ □Protein recovered/fraction

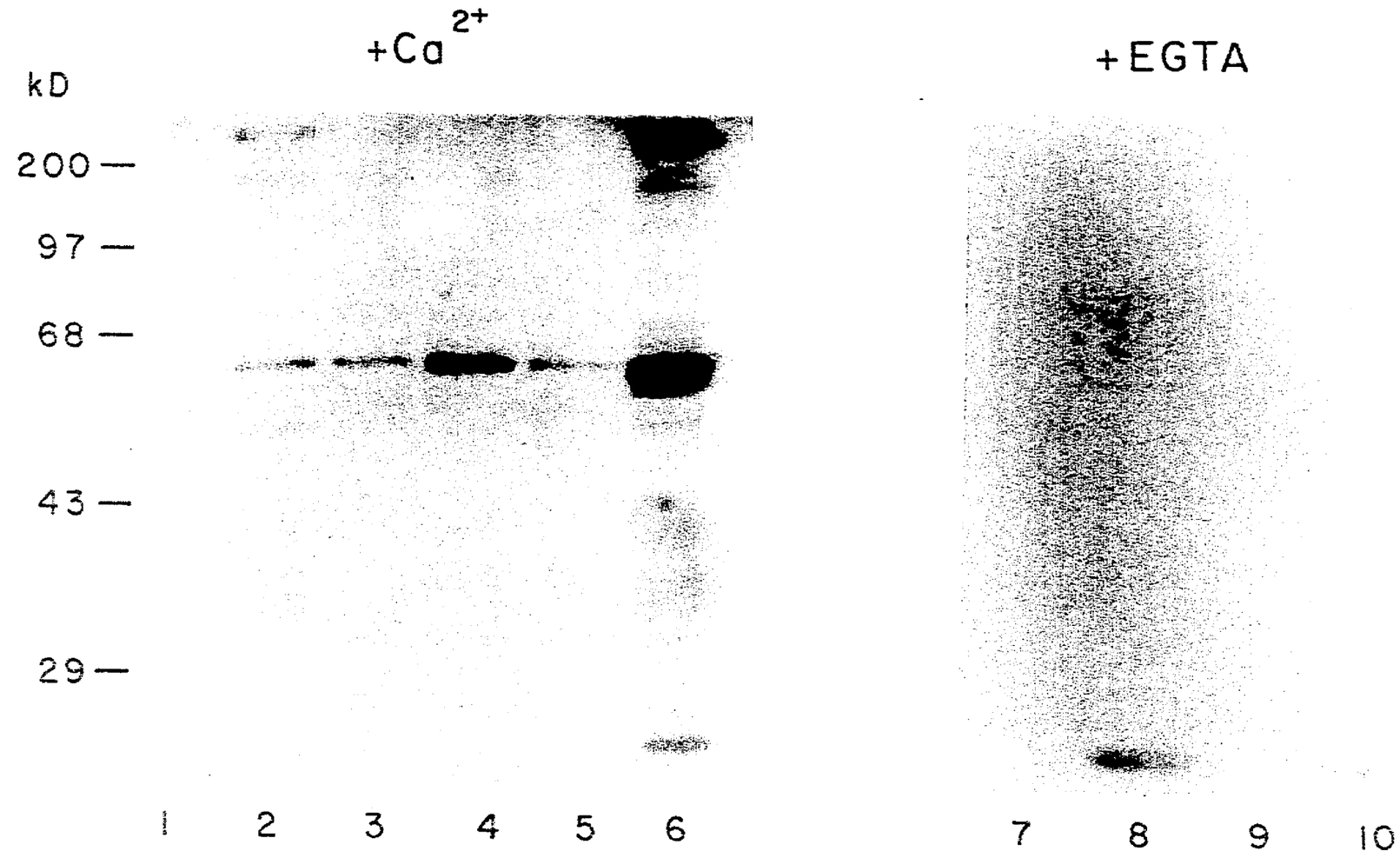
Text Fig. 4. Subcellular distribution of CaM in immature and adult rat testis. 20 day old and adult rat testis were homogenized with 0.25 M sucrose, 20 mM Hepes pH 7.2 using a Potter-Elvjhem homogenizer. Six subcellular fractions were obtained by differential centrifugation and assayed for CaM content. Pellets were resuspended in borate saline pH 8.4, containing 2 mM DTT, 1 mM EGTA, 0.1 % SDS, sonicated and heated as described for tissue and cell samples. DTT, EGTA and SDS were added to the cytosol to obtain 2 mM, 1 mM and 0.1% final concentrations, respectively. (1) 770 x g crude nuclear pellet (P_1). (2) 6 000 x g mitochondrial-lyzosomal fraction (P_2). (3) 12 500 x g heavy endoplasmic reticulum fraction (P_3). (4) 24 000 x g endoplasmic reticulum-plasma membrane fraction (P_4). (5) 150 000 x g light microsomal fraction (P_5). (6) final supernatant. Open circles CaM content in 20 day old rat testis. Filled circles CaM content in adult rat testis. Open squares percentage of the total protein recovered in each fraction from 20 day old rat testis. Filled squares protein recovered in each fraction from adult rat testis.


A
kD
200
116
92
66
45
E H I 2 3 4 5 6
B
kD
45
31
21
14
E H I 2 3 4 5 6

Text Fig. 5. Subcellular distribution of Ca^{2+}-dependent CaMBP in immature rat testis. 13 day old rat testis were homogenized with 0.25 mM sucrose, 20 mM Hepes and fractionated by differential centrifugation. Aliquots of 80 µg of protein of each fraction were resuspended with sample buffer and electrophoresed in 13 or 6.5 % PAGE. *Gels were overlaid with ^{125}I-CaM, 10^6 dpm per lane, in the presence of 2 mM $CaCl_2$ or 4 mM EGTA. Non-bound CaM was washed out and CaMBP were detected on Kodak film by autoradiography. (E) Electrophorus electricus* Ca^{2+}-CaMBP. (H) Testicular homogenate. (1) 770 x g crude nuclear pellet (P_1). (2) 6 000 x g mitochondrial-lyzosomal fraction (P_2). (3) 12 500 x g heavy endoplasmic reticulum (P_3). (4) 24 000 x g endoplasmic reticulum-plasma membrane pellet (P_4). (5) 150 000 x g microsomal fraction (P_5). (6) Cytosol. (A) 13 % (B) 6.5 % polyacrylamide gels.


kD
200
116
92
66
45
1 2
kD
200
116
92
66
45
E 3 4
kD
200
116
92
66
45
E 5 6
kD
92
66
45
31
21
14
E 7 8

Text Fig 6. Ca^{2+}-dependent CaMBP in isolated germ cells. Germinal cells were resuspended in sample buffer and separated on 13 and 6.5 % PAGE. Results obtained from 4 different experiments are shown. 6.5 % polyacrylamide gels: lane (1) Spermatogonia-Sertoli cell population from 12 day old rat testis. Lanes (2) and (3) Pachytene primary spermatocytes from 21 day old rat testis. Lane (E) *Electrophorus electricus* Ca^{2+}-CaMBP. Lanes (4) (5) and (6) Spermatids isolated from 38 day old rat testis. 13% polyacrylamide gels: Lane (7) Primary spermatocytes. Lane (8) Spermatids.


+Ca 2+
+EGTA
kD
200
97
68
43
29
1
2
3
4
5
6
7
8
9
10

Text Fig. 7. Ca^{2+}-dependent and Ca^{2+}-independent CaMBP in purified nuclei from primary spermatocytes. Pachytene primary spermatocytes, obtained from 25 day old rat testis, were fractionated to purify the nuclei as described in Materials and Methods. Aliquots of 80 μg of protein were separated on 10 % polyacrylamide gels. CaMBP were detected by overlay with ^{125}I-CaM in the presence of 2 mM $CaCl_2$ or 4 mM EGTA. Lane (1) Molecular weight standards. Lanes (2), (3), (8) and (10) purified nuclei. Lanes (4), (5), (7) and (9) supernatant obtained after centrifugation of the cell homogenate at 800 x g. Lane (6) Primary spermatocytes homogenate. Results from 2 different preparations of nuclei are shown.

RESULTADOS

Como puede observarse en el trabajo anterior, el 80% de la CaM testicular se recuperó en dos fracciones, la nuclear y la citosólica. La detección de diversas proteínas capaces de unir a CaM en forma dependiente de Ca^{2+} en las dos fracciones, sugirió que estos dos compartimentos estaban enriquecidos en CaM y en sus proteínas blanco.

La migración de CaM al núcleo y el rearreglo de las proteínas aceptoras de CaM relacionadas al esqueleto nuclear, observados en los hepatocitos inducidos a multiplicarse, plantearon la posibilidad de que algo semejante ocurriera durante la migración de CaM al núcleo de los espermatocitos primarios en estadios tardíos de la primera profase meiótica.

Se decidió entonces delimitar el tercer objetivo de esta tesis **aislar y purificar las proteínas blanco de CaM en los espermatocitos primarios**, a la purificación de las proteínas aceptoras de CaM de la matriz nuclear y compararlas con las aceptoras de CaM del citosol.

Para aislar las proteínas de la matriz nuclear, los ácidos nucléicos fueron hidrolizados con DNasa II y RNasa A. Los productos de la hidrólisis enzimática y parte de las proteínas asociadas a los ácidos nucléicos, fueron separadas por centrifugación a 800 x g y recuperadas en el sobrenadante. La pastilla fue extraída con NaCl 2 M y el material no solubilizado fué separado por centrifugación. Las proteínas de la pastilla o matriz nuclear fueron solubilizadas con Triton X-100, se agregó $CaCl_2$, a una concentración final de 500 μM y se adicionaron a una columna de Sefarosa-CaM. Las proteínas aceptoras de CaM dependientes de Ca^{2+} fueron eluídas empleando EGTA.

Para purificar las proteínas aceptoras de CaM del citosol fué necesario eliminar la CaM endógena presente en esta fracción. Para ello, la fracción soluble de los espermatocitos primarios, obtenida por centrifugación a 120 000 x g durante 60 min, fué fraccionado por cromatografía de intercambio iónico usando DEAE-celulosa. A la fracción eluída con 80 mM de NaCl se le adicionó $CaCl_2$, para obtener una concentración final de 500 μM y se usó como fuente de las proteínas aceptoras de CaM. Las proteínas capaces de unir CaM en forma Ca^{2+}-dependiente fueron purificadas por cromatografía de afinidad en una columna de Sefarosa-CaM.

Las proteínas aceptoras de CaM dependientes de Ca^{2+} de la matriz nuclear y del citosol fueron analizadas por electroforesis

en geles de poliacrilamida al 10%. Los resultados señalan la purificación parcial de seis proteínas aceptoras de CaM de la matriz nuclear, en contraste con la diversidad de proteinas aceptoras de CaM presentes en el citosol.

La matriz nuclear de los espermatocitos no muestró proteínas de alto peso molecular, como las del citosol. La semejanza del peso molecular de algunas de las proteínas aceptoras de CaM del citosol con proteínas definidas del citoesqueleto testicular, reforzaron la posibilidad de que estas proteínas estuvieran relacionadas al citoesqueleto de los espermatocitos primarios. Existen evidencias experimentales que señalan la participación de CaM en el control de la organización de los elementos del citoesqueleto a diversos niveles. Por una parte, aumentando la sensibilidad de los microtúbulos al Ca^{2+}, regulando así su polimerización-despolimerización. Por otra parte, mediante la fosforilación de proteínas asociadas a los microtúbulos, catalizada por la proteína cinasa dependiente de Ca^{2+}-CaM tipo II, modifica la interrelación entre los distintos elementos del citoesqueleto.

Con base en estos antecedentes y los resultados obtenidos se planteó el cuarto objetivo de esta tesis **definir si los espermatocitos primarios poseen actividad de proteína cinasa activada por Ca^{2+}-CaM.**

Para contestar esta pregunta se tomaron alícuotas de los núcleos purificados y del citoplasma de los espermatocitos primarios y se determinó su capacidad para fosforilar a la caseína copolimerizada con la acrilamida. Los geles fueron incubados con ^{32}P-ATP en presencia de Ca^{2+}-CaM o de EGTA y la incorporación de ^{32}P a la caseína fué determinada por autoradiografía.

Se detectó la presencia de una proteína cinasa con un peso molecular aproximado de 37kD, presente tanto en el núcleo como en el citoplasma de los espermatocitos primarios. La actividad de esta cinasa fué mayor en presencia de Ca^{2+}-CaM, pero también fué detectada en los geles incubados con EGTA.

En el citoplasma, exclusivamente, se detectaron dos bandas con un peso molecular aparente de 55 y 52 kD, capaces de fosforilar a la caseína solo en presencia de Ca^{2+}-CaM. El peso molecular de estas bandas y su dependencia de Ca^{2+}-CaM sugirió la presencia de la proteína cinasa tipo II en el citoplasma de los espermatocitos primarios.

La proteína cinasa asociada a la proteína de 37 kD podría deberse tanto a la actividad de la caseína cinasa tipo I como a una

cinasa dependiente de CaM tipo I. Ya que la actividad de la caseína cinasa I no es estimulada por Ca^{2+}-CaM y tampoco es inhibida por drogas anti-CaM, su participación pudo descartarse utilizando como fuente de enzima a las proteínas aceptoras de CaM dependientes de Ca^{2+} purificadas por cromatografía de afinidad tanto de la matriz nuclear como del citosol.

Estos dos grupos de proteinas mostraron una capacidad semejante para fosforilar a la caseína en presencia de Ca^{2+}-CaM. Cuando las proteínas aceptoras de CaM purificadas del citosol fueron incubadas en ausencia de sustratos exógenos, se observó que una banda de peso molecular mayor a 200 kD era fosforilada en presencia de Ca^{2+}-CaM y en presencia de Mg^{2+}-EGTA. Tanto la fosforilación de esta proteína como la fosforilación de caseína fue completamente inhibida con TFP a una concentración de 50 μM. El peso molecular de la proteína fosforilada recuerda el peso de las proteínas asociadas a los microtúbulos tipo 1 y 2, pero se necesita repetir su fosforilación y su separación en geles con menor porcentaje de acrilamida para definir su peso molecular y su identidad. También se observó que en presencia de Mg^{2+}-EGTA se puede fosforilar esta banda y otra de 18 kD. Estas observaciones señalan que la proteína cinasa del citosol y dos sustratos endógenos, son proteínas que unen a CaM en forma Ca^{2+}-dependiente.

A diferencia del citosol, la actividad de la proteína cinasa de la matriz nuclear de los espermatocitos primarios no fué totalmente inhibida por TFP. A la misma concentración, 50 μM, esta droga con actividad anti-CaM, solamente inhibió el 60% de la incorporación de ^{32}P a la caseína. Estos resultados podrían deberse a una hidrólisis parcial de la cinasa durante su purificación, ocasionando la pérdida del dominio que une a CaM y del dominio inhibidor presente en las proteínas activadas por Ca^{2+}-CaM. Ninguna de las proteínas aceptoras de Ca^{2+}-CaM purificadas de la matriz nuclear fué fosforilada por la proteína cinasa presente.

Los resultados sugieren que los espermatocitos primarios poseen dos tipos de cinasas activadas por Ca^{2+}-CaM con diferente localización intracelular. La proteína cinasa tipo II se localiza en el citosol, en tanto que la matriz nuclear presenta la proteína cinasa tipo I. Sin embargo se requiere realizar más experimentos para demostrar claramente esta interpretación.

Estas observaciones son presentadas a continuación en el trabajo "Ca^{2+}-Calmodulin-stimulated protein kinases in the nuclear matrix and cytosol from pachytene primary spermatocytes".

Ca^{2+}-Calmodulin-stimulated protein kinases in nuclear matrix and cytosol from pachytene primary spermatocytes.

Raquel Trejo.

Unidad de Investigación Médica en Enfermedades Oncológicas. Hospital de Oncología. Instituto Mexicano del Seguro Social. Apdo. Postal 12-1060. México D.F. México 03000.

Telephone numbers (525) 578-6174
(525) 627-6900 ext 4204 and 4323.
Fax (525) 761-0952.

Running title: Ca^{2+}-CaM-protein kinase activities in primary spermatocytes

ABSTRACT

Pachytene primary spermatocytes showed both casein kinase and Ca^{2+}-CaM dependent protein kinase activities. Purified nuclei and cytoplasm contained casein kinase activity associated to a 37-34 kD protein. Ca^{2+}-CaM-dependent protein kinase activity was detected in two cytoplasmic proteins M_r= 54 and 52 kD. To identify the protein kinase activities, Ca^{2+}-dependent calmodulin-binding proteins (Ca^{2+}-CaMBP) were purified by affinity chromatography from cytosol and nuclear matrix. The main cytosolic Ca^{2+}-CaMBP had an M_r= 90, 60, 54, 30, and 24 kD. These Ca^{2+}-CaMBP contained both Ca^{2+}-CaM protein kinase, possibly associated to the 60 and 54 kD polypeptides, and phosphorylatable substrates. A protein with molecular weight higher than 200 kD was phosphorylated in a Ca^{2+}-CaM dependent manner. 50 μM trifluoroperazine (TFP) completely block the Ca^{2+}-CaM kinase activity and the phosphorylation of endogenous substrates. The results indicate that the cytosolic protein kinase activity is related to the Ca^{2+}-CaM dependent protein kinase II. The major Ca^{2+}-CaMBP purified from nuclear matrix showed an apparent M_r= 66, 34, 31, 28, 27 and 18 kD. A protein kinase activity was present in these Ca^{2+}-CaMBP but was not stimulated by Ca^{2+}-CaM. However, 50 μM TFP inhibited 60% the activity. The protein kinase activity was possible associated to the 34 kD protein. No endogenous substrates copury with the enzyme.

Key words: Ca^{2+}-dependent calmodulin-binding proteins, spermatogenic cells, casein kinase, meiotic male cells, nuclear cytoskeleton,

INTRODUCTION

Progression of primary spermatocytes through meiosis was paralleled by an increase in calmodulin (CaM) content (Trejo *et al.*, submitted) and migration of CaM into the nucleus when pachytene and later stages of meiosis were reached (Moriya *et al.*, 1990). This situation is similar to the changes in concentration and localization of CaM in liver cells induced to proliferate (MacManus *et al.*, 1981; Piñol *et al.*, 1988). Quiescent and proliferatively activated liver cells showed differences in the nuclear matrix proteins able to bind CaM in a Ca^{2+}-dependent manner (Serratosa *et al.*, 1988; Pujol *et al.*, 1989; Bachs *et al.*, 1990). Binding of CaM to chromosomes as well as the intranuclear localization of CaM were also investigated (Fields and Shaper, 1988; Wong *et al.*, 1991; Portolés *et al.*, 1994).

Since CaM has been shown as an essential protein for cell proliferation and normal chromosome segregation (Davis *et al.*, 1986; Takeda and Yamamoto, 1987; Ohya and Anraku, 1989; Davis, 1992), identification of CaM targets in cells at division may further contribute to define the mechanism of CaM action in these processes. Pachytene primary spermatocytes were selected in this paper as experimental model system of proliferating cells to identify the targets of CaM in both nucleus and cytosol. To accomplish this, isolated nuclei from pachytene primary spermatocytes were further fractionated to purify those nuclear matrix proteins able to bind CaM in a Ca^{2+}-dependent

manner. Ca^{2+}-dependent calmodulin-binding proteins (CaMBP) were also purified from pachytene primary spermatocyte cytosol depleted from endogenous CaM. Since protein phosphorylation/dephosphorylation is one of the pathways by which CaM mediates Ca^{2+}-signalling of cell growth (for reviews see Lukas *et al.*, 1988; Kincaid, 1993; Takuwa *et al.*, 1995), the presence of Ca^{2+}-CaM-dependent protein kinase activities was screened in purified nuclei and cytoplasm of primary spermatocytes as well as in the Ca^{2+}-dependent CaMBP purified by affinity chromatography from nuclear matrix and from cytosol.

MATERIALS AND METHODS

Minimum essential medium (MEM), without calcium, supplemented with Earle salts, molecular weight protein standards (normal and prestained standards), acrylamide, bisacrylamide, Temed, molecular weight protein standards, sodium dodecyl sulfate (SDS), ammonium persulfate, Tris (hydroxymethyl) aminomethane, and synthide were purchased from Gibco, BRL. Collagenase form II was from Koch-Light Laboratories, Ltd,. Dextran T-500, CNBr-activivated Sepharose 4B and Ficoll were from Pharmacia Fine Chemicals. Sucrose, casein, bovine serum albumin (BSA) fraction V, soy bean trypsin inhibitor (SBTI), deoxyribunuclease II (DNase II) type IV, ribonuclease A (RNase), ethylenglycol-bis-(amino-ethyl ether)tetracetic acid (EGTA), DL-dithiothreitol (DTT), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), p-nitrophenylphosphate, phenyl sepharose, DEAE-cellulose, were from Sigma Chemicals. NaCl, KCl, KH_2PO_4, $MgCl_2$, $CaCl_2$, imidazol, sodium citrate, were from Merck. ^{32}P-ATP was from NEN.

CaM purification. CaM was purified from adult bovine testis by affinity chromatography on phenyl-sepharose as previously described by Gopalakrishna and Anderson (1978).

Isolation of nuclear matrix from pachytene primary spermatocytes. Primary spermatocytes at pachytene stage were obtained from 21-25 day old rat testes following the technique previously described (Alemán *et al*, 1978). Nuclei were purified following the technique of Birnie (1978) with some

modifications (Trejo *et al.*, submitted). To obtain the nuclear matrix, primary spermatocyte nuclear pellet was incubated with 250µg/ml of DNase I and 250 µg/ml of RNase A for 60 min at 4°C and extracted with 2 M NaCl as described (Bachs *et al.*, 1990).

Purification of Ca^{2+}-CaMBP from the nuclear matrix and cytosol obtained from primary spermatocytes. A) Nuclear matrix. The insoluble, high-salt nuclear extracted fraction was incubated 30 min at 4°C with 300 mM KCl, 0.2 mM $MgSO_4$, 50 mM Tris.HCl, pH 7.4, 1 mM PMSF, and 1% (w/v) Triton X-100. The solubilized material was adjusted to 500 µM $CaCl_2$ and applied to a CaM-sepharose column. Ca^{2+}-dependent CaM-binding proteins were obtained by elution with 2 mM EGTA as described (Bachs et al, 1990). B) Cytosolic CaMBP. The supernatant obtained after centrifugation of the cell homogenate at 800 x g, was centrifuged at 20 000 x g for 20 min and then at 120 000 x g for 60 min at 4°C. The final supernatant was the source of cytosolic CaMBP. Endogenous soluble CaM was eliminated by DEAE-cellulose chromatography as described (Miyakawa *et al.*, 1989). The fraction eluted with 0.08M NaCl was made 1 mM $CaCl_2$ and applied to a CaM-Sepharose column. Ca^{2+}-dependent CaMBP were eluted with 20 mH Hepes pH 7.0, 10 mM ß-mercaptoethanol, 200 mM NaCl and 2 mM EGTA. Eluted proteins from both nuclear matrix and cytosol were dialyzed with 20 mM Tris pH 7.0, and concentrated using polyethylen glycol 8 000. Purified Ca^{2+}-CaMBP were aliquoted and kept at -80°C.

Detection of protein kinase activity in substrate

polyacrylamide gels. Aliquots containing 80 μg of protein of purified nuclei and cytoplasm isolated from pachytene primary spermatocytes were used to detect protein kinase activity in casein containing gels as previously described (Miyakawa *et al.*, 1989) with some modifications. After extensive washing of the gel with 40 mM Hepes pH 7.5 for 6 hours, gels were incubated overnight at 4°C with 25 mM Hepes pH 7.5, 5 mM $MgCl_2$ and 25 μM ATP containing ^{32}P-ATP (20μCi) in the presence of 1 mM $CaCl_2$ and 4 μg/ml of CaM or with 1 mM EGTA in a heat sealed Scotchpak pouche. Gels were dried under vacuum and labeled bands were detected by autoradiography on Kodak film.

Protein kinase assay. Protein phosphorylation was determined using casein as substrate according to Miyakawa *et al* (1989). Aliquots of 50 or 25 μg protein of the Ca^{2+}-dependent CaMBP purified from cytosol and nuclear matrix, respectively, were incubated with 20 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM DTT. 1 mM EGTA or 1 mM $CaCl_2$ plus 10 μg CaM with and without 50 μM TFP. The final incubation volume was 0.25 ml. Reaction was started by addition of 2 mM ATP containing 2 μCi ^{32}P-ATP. After 10 min of incubation at 28°C, reaction was stopped by adding 2 ml of ice-cold 6% $HClO_4$. Precipitated proteins were pelleted by centrifugation of samples at 3 000 x g for 15 min at 4°C. Supernatant was discarded and pellet was washed twice with 2 ml of 6% $HClO_4$, and three times with 2 ml of absolute ethanol. Pellet was solubilized with SDS and counted in a Beckman LS 6000 SE counter.

Analysis of phosphorylated proteins. A) Ca^{2+}-CaM-dependent phosphorylation. Cytosolic Ca^{2+}-CaMBP, 50 μg protein, were incubated in the absence of exogenous substrates with 20 mM Tris, pH 7.0, 10 mM $MgCl_2$ supplemented with 1 mM $CaCl_2$, or with 1 mM $CaCl_2$ plus 10 μg CaM in the presence and in the absence of 50 μM TFP as described (Miyakawa *et al.*, 1989). B) Ca^{2+}-CaM-independent phosphorylation of endogenous substrates. Cytosolic and nuclear matrix Ca^{2+}-dependent CaMBP were incubated in the incubation medium described above but neither Ca^{2+}- nor CaM were added. In both experimental conditions protein phosphorylation was started by addition of 2 mM ATP plus 2 μCi ^{32}P-ATP. After 10 min incubation at 28°C, 2 ml of ice-cold 6% $HClO_4$ and 50 μg bovine serum albumin were added. After 15 min on ice, proteins were pelleted by centrifugation at 3 000 x g for 15 min and washed three times with 2 ml of ethanol. Pellet was solubilized with 50 μl of sample buffer and separated on SDS-10% PAGE.

Electrophoresis. Electrophoresis was run overnight at 40 volts using the buffer system of Laemmli (1970). Proteins were visualized with Coomassie blue or silver staining (Morrisey, 1981) .

Protein determination. Protein content of eluted samples from CaM-sepharose column was determined by absorbance at 280 nm using bovine serum albumine as standard. Purified nuclei and cytoplasm protein content was determined by Hartree method (1972).

RESULTS

Detection of protein kinase activity in purified nuclei and cytoplasm of primary spermatocytes. The presence of protein kinase activity in pachytene primary spermatocytes was screened using substrate gels. Purified nuclei and crude cytoplasm fractions were separated in casein-10% PAGE and incubated with ^{32}P-ATP either in the presence of Ca^{2+}-CaM or Mg^{++}-EGTA. In the presence of Ca^{2+}-CaM three proteins with an apparent M_r= 37, 55, and 52 kD showed protein kinase activity (Fig. 1). The 37 kD band showed a strong casein-kinase activity in both purified nuclei and cytoplasm incubated in the presence of Ca^{2+}-CaM, however, a difuse label associated to this band was also observed in the presence of Mg^{2+}-EGTA (Fig. 1). The proteins with M_r= 55 and 52 were only detected in the cytoplasm incubated with Ca^{2+}-CaM (Fig. 1). The molecular weight of these proteins resemble the molecular weight of Ca^{2+}-CaM protein kinase II subunits detected in many tissues (Cohen, 1988), but that have not been shown in rat pachytene primary spermatocytes. The protein kinase associated to the 37 kD band may be due to casein kinase activity which is independent of Ca^{2+}-CaM and has been observed in testis nuclei and cytosol (Yutani *et al.*, 1982; Singh and Huang, 1989). However, it may also reflect the activity of a Ca^{2+}-CaM kinase I (DeRemer *et al.*, 1992). To identify the type of Ca^{2+}-CaM-dependent protein kinase present in these cells, cytoplasm and nuclei were fractionated, cytosolic and nuclear matrix Ca^{2+}-CaMBP were

purified and their protein kinase activity was tested.

Ca^{2+}-dependent CaMBP purified by affinity chromatography from cytosol of pachytene spermatocytes. Crude cytoplasm obtained after centrifugation of cell homogenate at 800 x g for 10 min (twice), was centrifuged at 20 000 x g for 30 min and at 125 000 x g for 60 min at 4°C to separate membrane vesicles from soluble proteins. After endogenous CaM depletion by DEAE chromatography, several Ca^{2+}-dependent CaMBP were purified from pachytene spermatocyte cytosol on a CaM-Sepharose column. The apparent M_r of the major EGTA-eluted proteins was 90, 60, 54, 30 and 24 kD (Fig. 2 lane 1). Some minor proteins were also purified M_r= 120, 110. 84, 64, 38, 35, 34, and 18 kD.

Protein kinase activity in cytosolic Ca^{2+}-CaMBP. Ca^{2+}-CaMBP purified from pachytene primary spermatocyte cytosol showed protein kinase activity using casein as substrate. The activity shown in the presence of Mg^{++}-EGTA was stimulated 2 fold by Ca^{2+}-CaM and almost completely inhibited by 50 μM TFP (Fig. 3).

Ca^{2+}-dependent CaMBP in the nuclear matrix of primary spermatocytes. Purified nuclei of pachytene primary spermatocytes were fractionated to isolate the nuclear matrix as described (Bachs *et al.*, 1990). The major Ca^{2+}-CaMBP purified from this nuclear fraction had an apparent M_r = 66, 34, 30, 27, 26, and 18 kD. Two minor bands M_r= 55 and 52 were also detected (Fig. 2 lane 2).

Protein kinase activity in the nuclear matrix Ca^{2+}-CaMBP.

When Ca^{2+}-dependent CaMBP from nuclear matrix of primary spermatocytes were assayed for protein kinase activity, the incorporation of ^{32}P to casein in the presence of Mg^{++}-EGTA was three times higher than the level reached by the cytosolic protein kinase activity. Although Ca^{2+} addition stimulated in 30% the activity, it was not further increased by Ca^{2+}-CaM. However, an inhibition of 60% was produced by 50 μM TFP in the presence of Ca^{2+}-CaM (Fig. 4). These results could have been caused by partial hydrolysis of the enzyme during purification or by contamination with other protein kinases. The 34 kD protein may be a proteolytic product of the 37 kD nuclear kinase activity detected in susbrate gels incubated with Ca^{2+}-CaM. Although the molecular weight of the nuclear matrix Ca^{2+}-CaMBP resemble the molecular weight of casein kinase I and II, a possible contamination with these enzymes does not seem likely. Binding of these enzymes to Ca^{2+}-CaM has not been shown, they are not activated by Ca^{2+}-CaM and phosphorylation of somes substrates by casein kinase II was inhibited in the presence of Ca^{2+}-CaM (Hathaway and Traugh, 1982; Pinna, 1990; Issinger, 1993; Bosser *et al.*, 1993). Furthermore, activity of casein kinase I is lost by freezing (Hattaway and Traugh, 1982) and phosphorylation of casein was determined in frozen-thawed Ca^{2+}-CaMBP, stored at -80°C.

Phosphorylation of endogenous substrates. To determine whether Ca^{2+}-CaMBP purified from primary spermatocyte cytosol and nuclear matrix contained both protein kinase and endogenous

subtrates, these proteins were incubated with ^{32}P-ATP in the absence of exogenous substrates. In the presence of Ca^{2+} or with Ca^{2+}-CaM, cytosolic Ca^{2+}-CaMBP showed a radioactive labeled band of high molecular weight > 200 kD (Fig. 5 lane 1). The radioactive label was not observed when 50 μM TFP was added to the incubation mixture (Fig. 5 lane 2). However, the high-molecular weight protein as well as a 18 kD protein were phosphorylated in the presence of Mg^{2+}+ EGTA (Fig. 6 lane C). Nuclear matrix Ca^{2+}-CaMBP incubated with Mg^{2+}-EGTA in the absence of exogenous substrates did not show any labeled band (Fig. 6 lane N). These results indicate that substrates and protein kinase were not purified together from nuclear matrix and support the interpretation that the incorporation of ^{32}P to casein was not due to casein kinase activity.

DISCUSSION

The results obtained with substrate gels indicate that pachytene primary spermatocytes show casein kinase and two different Ca^{2+}-CaM dependent protein kinase activities. In the rat testis, casein kinase I is mainly localized in the nuclei and casein kinase II predominates in cytosol (Yutani *et al.*, 1982; Singh and Huang, 1989). It seems very likely that the casein kinase activity associated to the 37 kD protein in nuclei and cytoplasm fractions of pachytene spermatocytes, detected in substrate gels, may be related to casein kinase I. However, the activity of this enzyme was not further observed in purified Ca^{2+}-CaMBP. Casein kinase I nor casein kinase II activities are Ca^{2+} or Ca^{2+}-CaM dependent and trifluoroperazine has not been reported to inhibit any of them (reviewed by Hathaway and Traugh 1982; Pinna 1990; Issinger 1993). Furthermore, no labeled proteins were observed when nuclear Ca^{2+}-CaMBP were incubated with Mg^{2+} -EGTA.

Ca^{2+}-CaMBP purified from nuclear matrix showed protein kinase activity in the presence of Mg^{++}-EGTA or Ca^{2+}-CaM. Ca^{2+}-CaM did not stimulate casein phosphorylation but the inhibition observed in the presence of TFP suggests that part of the enzyme was able to interact with Ca^{2+}-CaM. Activation of the kinase by partial proteolysis during isolation procedure could cause this result. This explanation is supported by the differences in molecular weight of the labeled band in the casein-polyacrylamide gels incubated with Ca^{2+}-CaM and the

proteins purified by affinity chromatography. The 34 kD polypeptide may be responsible for the protein kinase activity. These results suggest the presence of Ca^{2+}-CaM kinase I in the nuclear matrix of pachytene primary spermatocytes.

The cytosolic Ca^{2+}-CaMBP showed a protein kinase activity enhanced two-fold by Ca^{2+}-CaM and almost completely inhibited by 50 μM TFP. It seems very likely that the 60 and 54 kD proteins purified on CaM-Sepharose column are responsible for the cytosolic protein kinase activity. This interpretation is supported by the demonstration of mRNA for ß, , and isoforms of Ca^{2+}-CaM-dependent protein kinase II in rat testis (Tobimatsu and Fujisawa, 1989). These polypeptides copurify with other proteins susceptible to phosphorylation and able to interact with CaM in a Ca^{2+}-dependent manner. A high molecular weight Ca^{2+}-CaMBP (>200 kD) was phosphorylated by the cytosolic kinase in a Ca^{2+}-CaM dependent manner and the phosphorylation was inhibited by TFP. This band and a protein with a molecular weight of about 20 kD were phosphorylated in the presence of Mg^{++}-EGTA. Autophosphorylation of Ca^{2+}-CaM-dependent protein kinase II in the presence of Mg^{++}-EGTA produced an "hyperactive" enzyme (Lickteig *et al.*, 1988). It seems very likely that the conditions used in this experiment induced the Ca^{2+}-CaM-independent autophosphorylation of the enzyme and allowed the phosphorylation of these two substrates. The molecular weight of these proteins resembles the molecular weight of microtubule associated proteins able to bind CaM (Lee and Wolff, 1984) and

of myosin light chain, respectively (reviewed by Wiche, 1989). MAP-2 phosphorylation by Ca^{2+}-CaM protein kinase II has been observed in other cell types (Goldering *et al.*, 1983). *in vitro* studies have shown that phosphorylated MAP-2 modified microtubule assembly/disassembly and decreased its interaction with actin (for review see Gratzer and Baines, 1988; Wiche, 1989). On the other hand, cells with high CaM content due to overexpression of CaM gene, showed changes not only in microtubules but also in the organization of microfilaments and intermediate filaments as well as a decrease in the stability of ß-tubulin mRNA (Rasmussen and Means, 1992). These effects of CaM may have special physiological meaning in pachytene primary spermatocytes since these cells showed both the highest CaM content on cell basis (Trejo *et al.*, submitted) and Ca^{2+}-CaM protein kinase activity. One of the major cytosolic Ca^{2+}-CaMBP detected in this paper M_r= 90 resemble the molecular weight of a subunit of the cytoplasmic dynein (Neely *et al.*, 1990). Whether this Ca^{2+}-CaMBP is related to the microtubule-associated motor protein remains to be defined.

Nuclear matrix of several cell types is mainly constituted by extensive granular and fibrous proteins, by residual nuclear envelope proteins and by residual highly condensed nucleoli. Proteins with molecular weight higher than 100 kD predominate over proteins ranging between 80-60 kD, low molecular weight proteins reached the minimal proportion (Berezney and Coffey, 1975). Ca^{2+}-CaMBP purified from pachytene

spermatocyte nuclear matrix did not include high molecular weight Ca^{2+}-CaMBP similar to those found in hepatocyte nuclear cytoskeleton (Serratosa *et al.*, 1988; Bachs *et al.*, 1990). Nuclear matrix Ca^{2+}-CaMBP from primary spermatocytes was mainly constituted by proteins with a molecular weight of 34, 31, 27, 28 and 18 kD. Although, a 66 kD protein was also clearly observed and two minor proteins of 55 and 52 kD were also detected. The 66 kD Ca^{2+}-CaMBP purified from the nuclear matrix pachytene primary spermatocytes resemble the molecular weight of one residual lamina polypeptide previously detected in liver cell nuclear matrix proteins (Berezney and Coffey, 1975).

Gene expression and protein synthesis undergo remarkable changes throughout differentiation of the male germinal epithelium. Testis specific genes are triggered at pachytene and early spermatid stages of germ cell differentiation (reviewed by Eddy *et al.*, 1993; Hecht 1993). However, gene expression is abolished during spermatid maturation (Kierzebaum and Tres 1975), and translation of stored mRNA by spermatids has also been shown (reviwed by Schäfer *et al.*, 1995). CaM has been involved in the control of gene expression through phosphorylation of transcription factors by Ca^{2+}-CaM kinase II (Kapiloff *et al.*, 1991; Dash *et al.*, 1991; Sheng *et al.*, 1991; Wegner *et al.*, 1992; Cornellussen *et al.*, 1994) and by binding to proteins related to RNA processing and splicing (Bosser *et al.*, 1995; Bachs *et al.*, 1994). Whether the low molecular weight Ca^{2+}-CaMBP isolated from the nuclear matrix of pachytene

primary spermatocytes are related to these functions is currently investigated.

BIBLIOGRAPHY

Alemán V, Trejo R, Morales E, Hernández-Jáuregui P, and Delhumeau-Ongay G (1978): A simple and rapid technique to isolate enriched populations of spermatocytes and spermatids from the immature rat testis. J Reprod Fert 56: 67-75.

Bachs O, Lanini L, Serratosa J, Coll MJ, Bastos R, Aligué R, Rius E, Crafoli E (1990): Calmodulin-binding proteins in the nuclei of quiescent and proliferatively activated rat liver cells. J Biol Chem 265:18595-18600.

Bachs O, Agell N, Carafoli E (1994): Calmodulin and calmodulin-binding proteins inthe nucleus. Cell Calcium 16: 289-296.

Berezney R, Coffey DS (1976): The nuclear matrix: isolation, structure and functions. Adv Enzyme Regul 14:63-100.

Birnie GD (1978): Isolation of nuclei from animal cells in culture. In Stein g, Stein J, Kleinsmith LJ (eds): "Methods in Cell Biology". Vol XVIII. Academic Press, New York. Chap.2 pp13-26.

Bosser R, Aligué R, Guerini D, Agell N, Carafoli E, Bachs O (1993): Calmodulin can modulate protein phosphorylation in rat liver cells nuclei. J Biol Chem 268:15477-15483.

Bosser R, Faura M, Serratosa J, Renau-Piqueras J, Pruschy M, Bachs O (1995) Phosphorylation of rat liver heterogeneous nuclear ribonucleoproteins A2 and C can be modulated by calmodulin. Mol Cell Biol 15:661-670.

Cohen P (1988): The calmodulin-dependent multiprotein kinase.

In Cohen P, Klee B (eds): "Molecular Aspects of Cellular Regulation" Vol 5 Calmodulin.Elsevier Science Publishers B.V., Netherlands. Chap. 8 pp 145-194.

Cornellussen B, Holm M, Waltersson Y, Onions J, Hallberg B, Thornell A, Grundström T (1994): Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains. Nature 368:760-764.

Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER (1991): cAMP response element-binding proteins is activated by Ca^{2+}/calmodulin-as well as cAMP-dependent protein kinase. Proc Natl Acas Sci USA 88:5061-5065.

Davis TN, Urdea MS, Masiarz FR, Thorner J (1986): Isolation of the yeast calmodulin gene: calmodulin is an essential protein, Cell 47:423-431.

Davis TN (1992): A temperature-sensitive calmodulin mutant loses viability during mitosis. J Cell Biol 118:607-617.

DeRemer MF, Saeli RJ, Edelman AM (1992): Ca^{2+}-calmodulin-dependent protein kinases Ia and Ib from rat brain I. Identifiaction, purification, and structural comparisons. J Biol Chem 267:13460-13465.

Eddy EM, Welch IE, O'Brien DA (1993): Gene expression during spermatogenesis. In de Kretser (ed): "Molecular Biology of the Male Reproductive System". Academic Press, San Diego CA, pp 181-232.

Fields AP, Shaper JH (1988): A major 62-kD intranuclear matrix polypeptide is a component of metaphase chromosomes. J

Cell Biol 107:833-840.

Goldering JR, González B, McGuire JS Jr, DeLorenzo RJ (1983): Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associaated proteins. J Biol Chem 258:12632-12640.

Gopalakrishna R, Anderson WB (1982): Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem Biophys Res Commun 194:830-836.

Gratzer WM, Baines AJ (1988): Calmodulin and cytoskeletal function. In Cohen P, Klee CB (eds): "Molecular aspects of cellular regulation" Vol 5 Calmodulin. Elsevier Science Publishing Company, Netherlands. Chap. 15 pp 329-340.

Hattaway GM, Traugh JA (1982): Casein kinases - Multipotential protein kinases. Curr Top Cell Regul 21:101-127.

Hartree EF (1973): Determination of protein. A modification of the Lowry method that gives a linear photometric response. Analyt Biochem 48:422-426.

Hecht NB (1993): Gene expression during male germ cell development. In Desjardins C, Ewing LL (eds): "Cell and Molecular Biology of the Testis". Oxford University Press, New York, NY, pp 400-432.

Issinger O-G (1993): Casein kinases: pleiotropic mediators of cellular regulation. Pharac Ther 59:1-30.

Kapiloff MS, Mathus JM, Nelson CA, Lin CR, Rosenfeld MG (1991):

Calcium/calmodulin-dependent protein kinase mediates a pathway for transcriptional regulation. Proc Natl Acad Sci USA 88:3710-3714.

Kierszenbaum AL, Tres LL (1975): Structural and transcriptional features of the mouse spermatid genome. J Cell Biol 65:258-270.

Kincaid R (1993): Calmodulin-dependent protein phosphatases from microorganisms to man. A study in structural conservatism and biological diversity. In Shenolikar S, Nairn AC (eds): "Advances in Second Messenger and Phosphoprotein Research". Vol. 27. Raven Press, Ltd., New York. pp 1-23.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee YC, Wolff J (1984): Calmodulin binds to both microtubule-associated protein 2 and proteins. J Biol Chem 259:8041-8044.

Lickteig R, Shenolikar S, Denner L, Kelly PT (1988): Regulation of Ca^{2+}/calmodulin-dependent protein kinase II by Ca^{2+}/calmodulin-independent autophosphorylation. J Biol Chem 263:19232-19239.

Lukas TJ, Haiech J, Lau W, Craig TA, Zimmer WE, Shattuck RL, Shoemaker MO, Watterson DM (1988): Calmodulin and calmodulin-regulated protein kinases as transducers of intracellular calcium signals. Cold Spring Harbor Symp

Quant Biol LIII:185-193.

MacManus JP, Braceland BM, Rixon RH, Whitefield JF, Morris HP (1981): An increase in calmodulin during gtowth of normal and cancerous liver in vivo FEBS Lett 133:99-102.

Miyakawa T, Oka Y, Tsuchiya E, Fukui S. (1989): *Saccharomyces cerevisiae* protein kinase dependent on Ca^{2+} and calmodulin. J Bacteriol 171:1417-1422.

Moriya M, Katagiri C, Yagi K (1993): Immuno-electron microscopic localization of calmodulin and calmodulin-binding proteins in the mouse germ cells during spermatogenesis and maturation. Cell Tissue Res 271:441-451.

Morrissey JH (1981): Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Analyt Biochem 117:307-310.

Neely MD, Erickson HP, Boeckelheide K (1990): HMW-2, the Sertoli cell cytoplasmic dynein from rat testis, is a dimer composed of nearly identical subunits. J Biol Chem 265:8691-8698.

Ohya Y, Anraku Y (1989): A galctose-dependent *cmd1* mutant of *Saccharomyces cerevisiae*: involvement of calmodulin in nuclear division. Curr Genet 15:113-120.

Pinna LA (1990): Casein kinase 2: an 'eminence grise' in cellular regulation. Biochim Biophys Acta 1054:267-284.

Piñol MR, Berchtold MW, Bachs O, Heizmann CW (1988): Increased calmodulin synthesis in the pre-replicative phase of rat

liver regeneration. FEBS Lett 231:445-450.

Portolés M, Faura M, Renau-Piqueras J, Iborra FJ, Saez R, Guerri C, Serratosa J, Rius E, Bachs O. (1994): Nuclear calmodulin/62 kDa calmodulin-binding protein complexes in interphasic and mitotic cells. J Cell Sci 107:3601-3614.

Pujol MJ, Soriano M, Aligué R, Carafoli E, Bachs O. (1989): Effects of α-adrenergic blockers on calmodulin association with the nuclear matrix of rat liver cells during proliferative activation. J Biol Chem 264:18863-18865.

Rasmussen CD, Means AR (1992): Increased calmodulin affects cell morphology and mRNA levels of cytoplasmic protein genes. Cell Motil Cytoskel 21:45-57.

Schäfter M, Nayernia K, Engel W, Schäfer U (1995): Translational control in spermatogenesis. Devel Biol 172:344-352.

Serratosa J, Pujol MJ, Bachs O, Carafoli E (1988): Rearrangement of nuclear calmodulin during proliferative liver cell activation. Biochem Biophys Res Commun 150:1162-1169.

Sheng M, Thompson MA, Greenberg ME (1991): CREB: A Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427-1430.

Singh TJ, Huang K-P (1989): Glycogen synthase (casein) kinase-1: Tissue distribution and subcellular localization. FEBS Lett 190:84-88.

Takeda, T Yamamoto M (1987): Analysis and in vitro disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. Proc Natl Acad Sci USA 84: 3580-3584.

Takuwa N, Zhou W, Takuwa Y (1995): Calcium, calmodulin and cell cycle progression. Cell Signal 7:93-104.

Tobimatsu T, Fujisawa H (1989): Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem 264:17907-17912.

Trejo R, Turner R, Delhumeau G, Dedman JR (1996): Characterization of the calmodulin-acceptor protein system in developing rat testis (submitted)

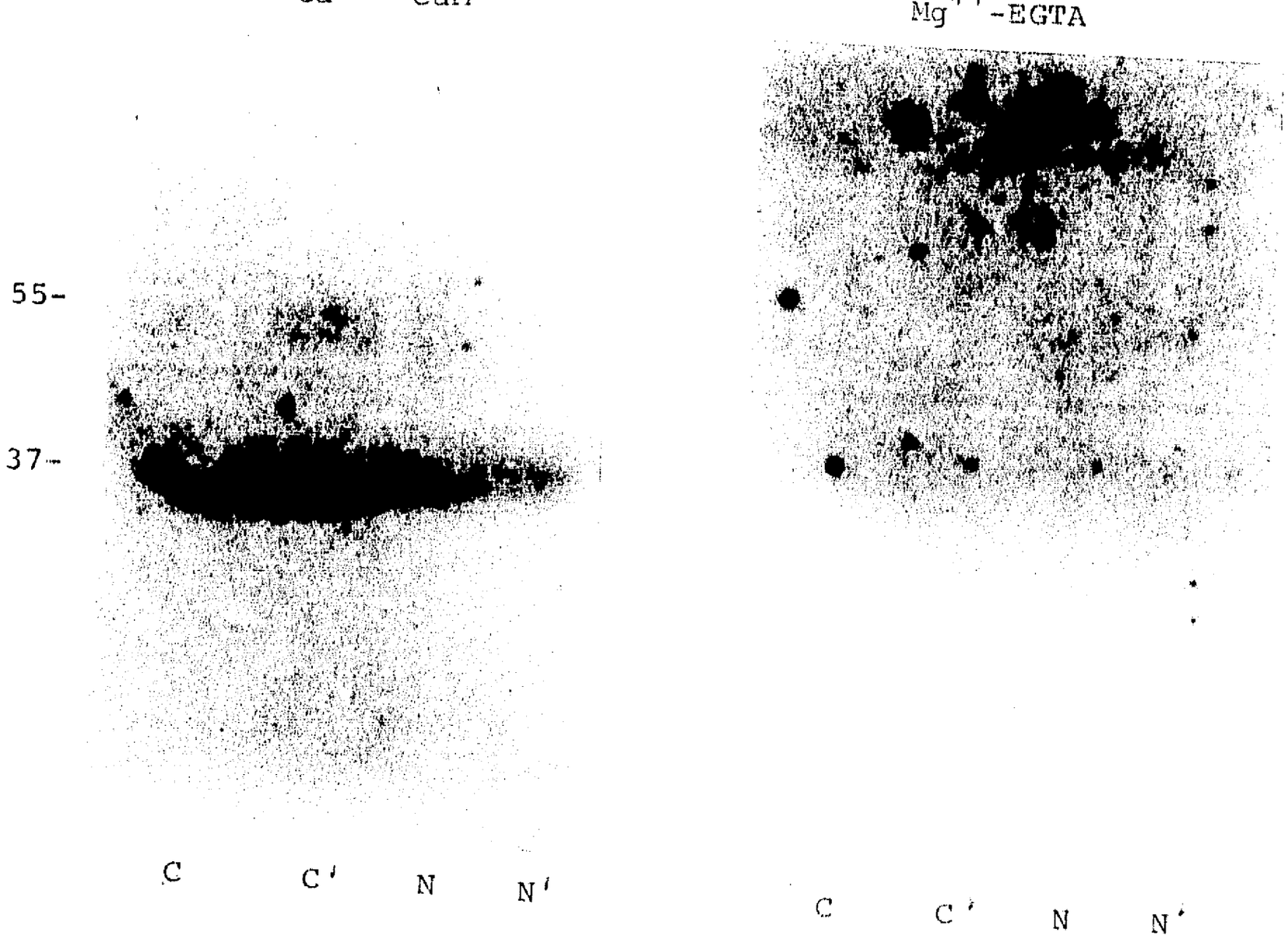
Wegner M, Cao Z, Rosenfeld MG (1992): Calcium-regulate4d phosphorylation within the leucine zipper of C/EBPß. Science 256:370-373.

Wiche G (1989): High-M_r microtubule-associated proteins: properties and funcitons. Biochem J 259:1-12.

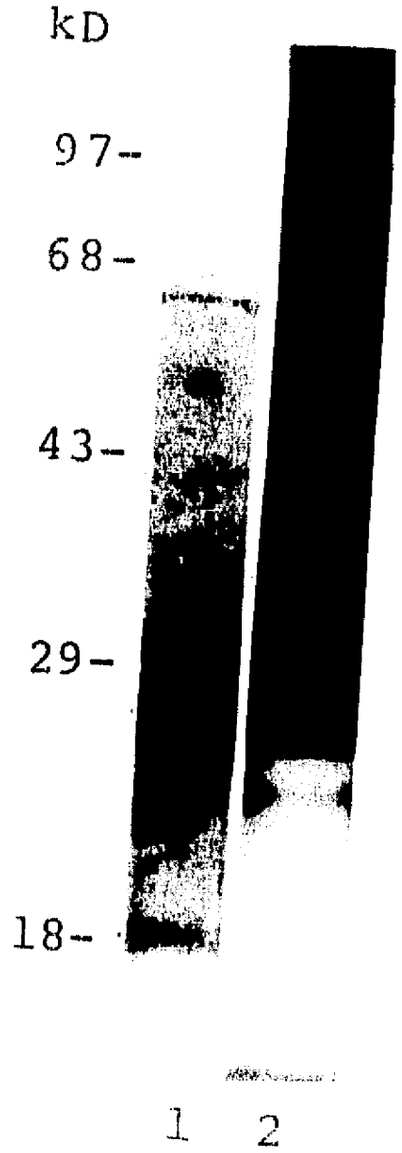
Wong ECC, Saffitz JE, McDonald JM (1991): Association of calmodulin with isolated nuclei from rat hepatocytes. Biochem Biophys Res Commun 181:1548-1556.

Yutani Y, Tei Y, Yukioka M, Inou A (1982): Occurrence of NI and NII type of protein kinases in the nuclei from various tissues of the rat. Arch Biochem Biophys 218:409-420.

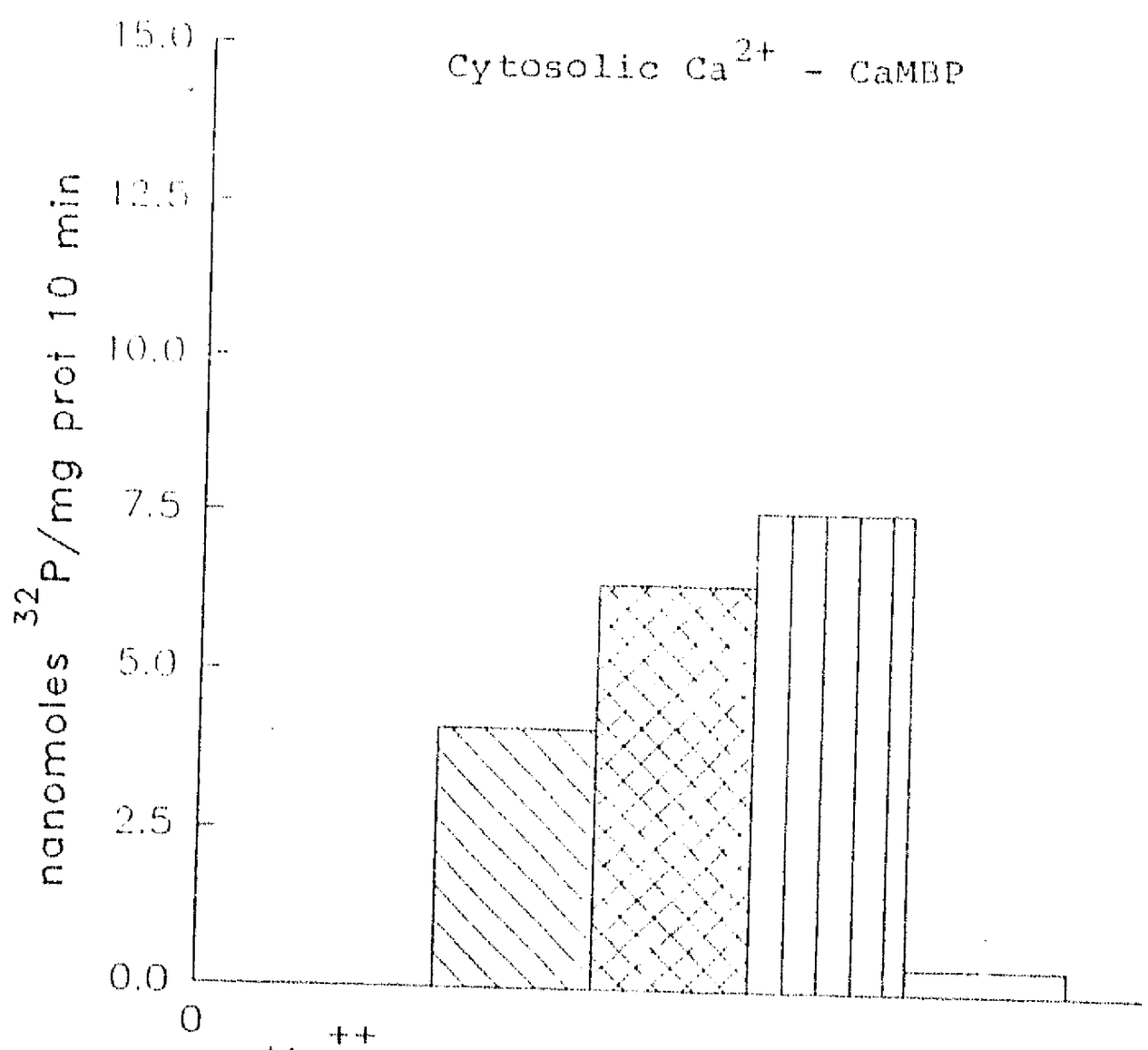
Protein Kinase in Pachytene Primary Spermatocytes



Text Fig 1 Detection of protein kinase activities in pachytene primary spermatocytes. Pachytene spermatocytes, isolated from 25 day old rats, were homogenized with 0.25 M sucrose, 20 mM imidazol pH 7.2, 1 mM EDTA and soy bean trypsin inhibitor 100 µg/ml. Cell homogenate was centrifuged at 800 x g for 10 min at 4°C, twice. The supernatant was saved and without further fractionation was used to detect protein kinase activity. Removal of cytoplasmic proteins from crude nuclear pellet was carried out by hipotonic treatment and centrifugation on 0.8 M sucrose (Birnie et al., 1978; Trejo et al., submitted). Aliquots of 80 µg of protein of cytoplasm and purified nuclei were separated on casein-10 % PAGE. After electrophoresis gels were incubated overnight at 4°C with ^{32}-P-ATP in the presence of Ca^{2+}-CaM or with Mg^{++}- 2 mM EGTA. Protein kinase activity was determined by autoradiography of dried gels. Results from 2 different preparations are shown. (C) and (C') cytoplasm. (N) and (N') purified nuclei.


kD
97-
68-
43-
29-
18-
1
2

Text Fig 2. Cytosolic and nuclear matrix Ca^{2+}-dependent CaMBP. Soluble fraction of primary spermatocytes obtained by centrifugation at 120 000 x g for 60 min at 4°C was the source of Ca^{2+}-CaMBP. Endogenous CaM was removed by DEAE chromatography. Proteins eluted with 0.08 M NaCl were further purified on CaM-Sepharose column equilibrated with $CaCl_2$. Ca^{2+}-dependent CaMBP were obtained by elution with 2 mM EGTA. Nuclear matrix proteins were isolated from pachytene primary spermatocytes after hydrolysis of nucleic acids with DNase II plus RNase A and extraction with 2 M NaCl as described in Materials and Methods. Solubilized proteins were adjusted to 500 μM $CaCl_2$ and onto CaM-Sepharose column. Ca^{2+}-dependent CaMBP were eluted with 2 mM EGTA. Aliquots of μg protein were separated in 10% PAGE and stained with silver (Morrisey, 1981). Lane 1 Ca^{2+}-CaMBP from nuclear matrix. Lane 2 Ca^{2+}-CaMBP from cytosol.



Mg_{2+}	+	+	+	+
Ca	−	+	+	+
CaM	−	−	+	+
TFP	−	−		+

Fig. 3. Protein kinase activity of cytosolic Ca^{2+}-CaMBP. Aliquots of 50 μg protein of affinity purified cytosolic Ca^{2+}-dependent CaMBP were incubated with 20 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 100 μg of casein. When indicated 1 mM $CaCl_2$ or 10 μg CaM or 50 μM TFP were added. Reaction was started by addition of 2 mM ATP containing 2 μCi ^{32}P-ATP. After 10 min of incubation at 28°C, 2 ml of ice cold $HClO_4$ was added. Samples stand on ice for 15 min and precipitated proteins were recovered by centrifugation at 3 000 x g for 15 min at 4°C. Pellet was washed twice with $HClO_4$ and three times with 2 ml of absolute ethanol. Pellet was solubilized with 100 μl of 1% SDS and 2 ml of scintillation cocktail and counted. Data shown are the means of 2 different experiments with determinations in duplicate.

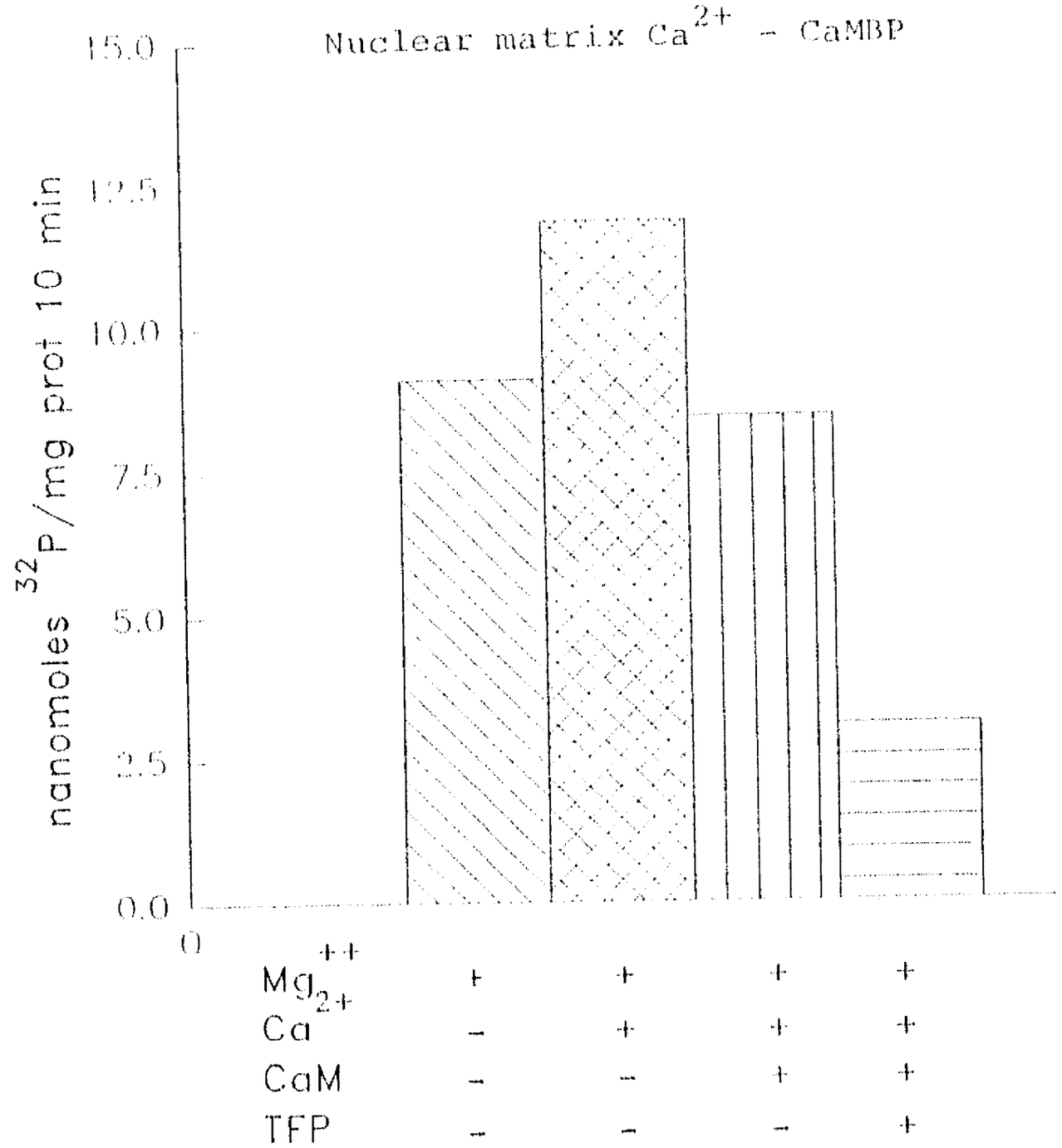

Nuclear matrix Ca^{2+} - CaMBP
nanomoles ^{32}P/mg prot 10 min
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
Mg_{2+} ++ + + + +
Ca - + + +
CaM - - + +
TFP - - - +

Fig. 4 Protein kinase activity of nuclear matrix Ca^{2+}-CaMBP. Aliquots of 25 μg protein of nuclear matrix Ca^{2+}-dependent CaMBP were incubated in the medium and conditions described in Fig. 3. Data shown are the mean of duplicate determinations of one experiment.

Ca^{2+}-CaM-dependent phosphorylation of cytosolic substrates

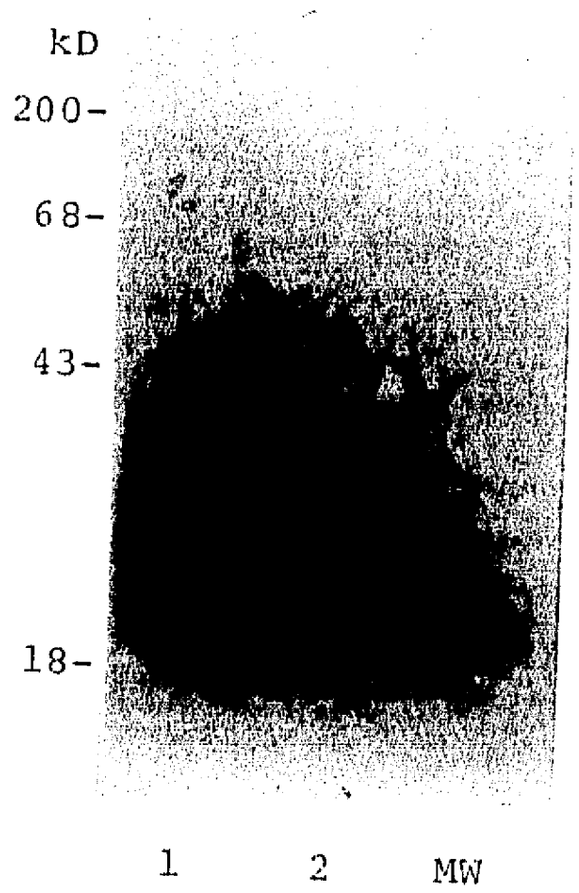


Fig 5. Ca^{2+}-CaM-dependent phosphorylation of endogenous substrates. Aliquots of 50 μg protein of cytosolic Ca^{2+}-dependent CaMBP were incubated as described in Fig. 3, except that casein was not added. Reaction was started with 2 mM ATP plus 2 μCi ^{32}P-ATP. After 10 min of incubation at 28°C, reaction was stopped with 2 ml of ice-cold 6% $HClO_4$. Bovine serum albumin was added, 50 μg, as carrier. Precipitated proteins were washed with $HClO_4$ and absolute ethanol as described in Materials and Methods and solubilized with 50 μl of sample buffer, heated 2 min at 95°C and separated on SDS-PAGE. Lane (1) Ca^{2+} + Mg^{2+}. Lane (2) Ca^{2+}+ Mg^{2+} + CaM + 50 μM TFP.

Ca^{2+}-CaM-independent phosphorylation of endogenous substrates

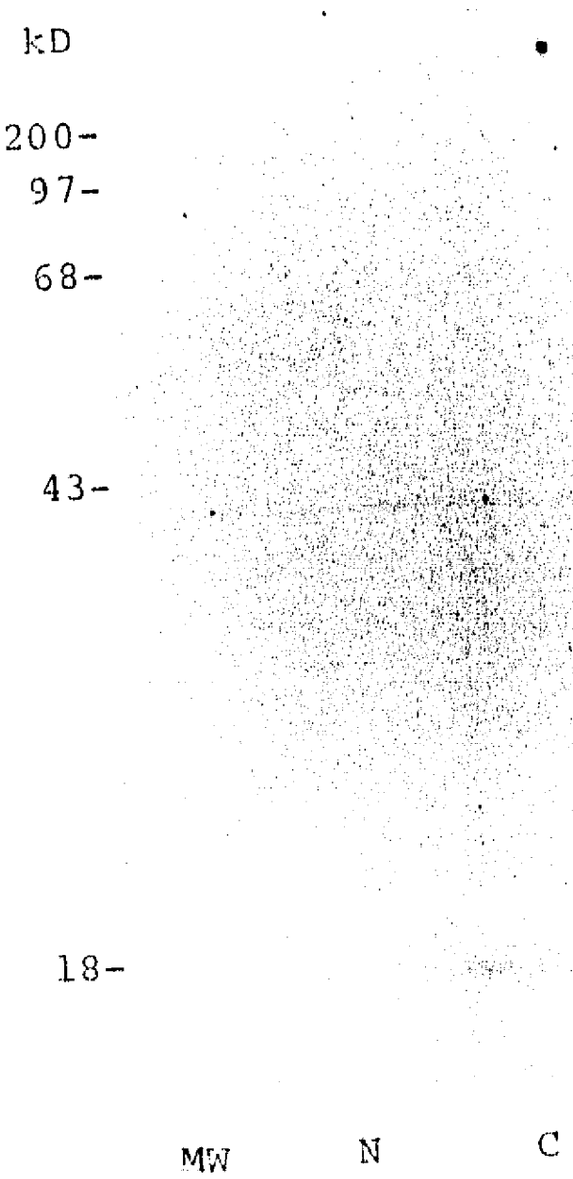


Fig. 6. Ca^{2+}-CaM independent phosphorylation of endogenous substrate. Cytosolic and nuclear matrix Ca^{2+}-dependent CaMBP were incubated with 20 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA and 2 mM ATP plus 2 μCi ^{32}P-ATP. After 10 min of incubation at 28°C, 2 ml of 6% $HClO_4$ and 50 μg of bovine serum albumin were added. Pellet was washed and resuspended with sample buffer as described in Fig. 5. (PM) molecular weight protein standards. (N) nuclear matrix Ca^{2+}-dependent CaMBP. (C) Cytosolic Ca^{2+}-dependent CaMBP.

RESULTADOS

En el espermatozoide la reacción acrosomal, la movilidad flagelar y la fertilización del óvulo son procesos dependientes de Ca^{2+}. Para la expresión de la reacción acrosomal se requiere un aumento en la permeabilidad de la membrana plasmática y acrosomal al Ca^{2+}. En el espermatozoide de los mamíferos, esta característica se alcanza durante el proceso conocido como capacitación. La fusión membranal y formación de vesículas mixtas de membrana plasmática y membrana acrosomal externa y la liberación del contenido acrosomal, son dependientes de Ca^{2+} y son uno de los resultados de la capacitación. La relevancia de los flujos de Ca^{2+} en la reacción acrosomal puede ser puesta de manifiesto utilizando un ionóforo de Ca^{2+} como A23187, que dispara la fusión membranal y la liberación del contenido acrosomal en espermatozoides no capacitados. Sin embargo, el papel de CaM como mediadora de los efectos del Ca^{2+} en este proceso no se ha definido.

Para alcanzar el quinto objetivo de esta tesis **definir si durante la capacitación y reacción acrosomal del espermatozoide se pueden identificar a las proteínas aceptoras de CaM que participan en estos procesos** primero se estudió la localización de CaM durante la capacitación *in vitro* del espermatozoide para definir si los cambios en la permeabilidad al Ca^{2+} que se presentan durante este proceso se asociaban con cambios en la localización de CaM. Se comparó la localización de CaM en espermatozoides no capacitados con la distribución de CaM en el espermatozoide que había sufrido la reacción acrosomal. Ya que el A23187 induce la entrada de Ca^{2+}, su adición al medio de incubación permite sincronizar a una población de espermatozoides en la realización de los eventos dependientes de Ca^{2+} y estudiar las modificaciones en el sistema Ca^{2+}-CaM-proteinas aceptoras que se presentaran durante la reacción acrosomal.

Se observaron cambios en la localización de CaM asociados a la progresión de la reacción acrosomal, expresada después de la capacitación o por adición de A23187. De una localización difusa en el espermatozoide no capacitado, evolucionó a una localización más definida en el acrosoma y en el flagelo. Aunque parte de la CaM se liberó junto con las vesículas acrosomales, otra parte quedó asociada a la región subecuatorial del núcleo y al flagelo en los espermatozoides que ya perdieron el acrosoma. Los resultados se muestran en el trabajo "Changes in calmodulin compartmentalization

throughout capacitation and acrosome reaction in guinea pig spermatozoa" realizado en colaboración con la Dra. Adela Mújica y publicado en la revista Molecular Reproduction and Development 26:365-376 (1990).

Los cambios en la localización de CaM sugirieron que los dominios del espermatozoide podrían tener proteínas aceptoras distintas para esta proteína. La asociación de CaM al acrosoma y su liberación junto con las vesículas de la reacción acrosomal, señalaron la importancia de identificar a las proteínas aceptoras de CaM en este dominio del espermatozoide. La localización de CaM en la región subecuatorial sugería que esta zona, involucrada en el contacto óvulo-espermatozoide podría estar especialmente enriquecida en proteínas aceptoras de CaM.

Con base en estas observaciones, se decidió recuperar las vesículas membranales derivadas de la reacción acrosomal. Para ello, se indujo la reacción acrosomal en una población de espermatozoides con A23187. También se aislaron y purificaron las cabezas de los espermatozoides que habían sufrido la reacción acrosomal y se usaron para obtener las proteínas de la porción subecuatorial e identificar a las proteínas que unen a CaM en esta región. La presencia de las proteínas aceptoras de CaM en todas las fracciones obtenidas del espermatozoide, se realizó por la técnica de overlay con CaM biotinilada o con CaM nativa, usando anticuerpos anti-CaM y un segundo anticuerpo acoplado a peroxidasa. El perfil de proteínas capaces de unir a CaM de estas fracciones se comparó con el patrón obtenido usando las membranas del espermatozoide intacto.

Se observó que el espermatozoide contiene numerosas proteínas que unen a CaM tanto en forma dependiente como independiente de Ca^{2+}, localizadas tanto en las vesículas derivadas de la reacción acrosomal como en las proteínas solubilizadas de la región subecuatorial de la cabeza del espermatozoide que ha experimentado la reacción acrosomal.

Observaciones similares han sido descritas por distintos autores empleando técnicas afines, lo que sugiere que el método empleado tiene limitaciones en la detección de las proteínas aceptoras de CaM dependientes de Ca^{2+}, posiblemente debido a un requerimiento de concentraciones específicas de Ca^{2+}.

Los resultados fueron reunidos en el trabajo "Calmodulin binding proteins in the membrane vesicles released during the acrosome reaction and in the perinuclear material in isolated

acrosome reacted sperm heads" realizado en colaboración con Enrique Hernández, Ana María Espinoza, Arturo González y Adela Mújica y fueron publicados en la revista Cell and Tissue 26: 849-865 (1994).

MOLECULAR REPRODUCTION AND DEVELOPMENT 26:366–376 (1990)

Changes in Calmodulin Compartmentalization Throughout Capacitation and Acrosome Reaction in Guinea Pig Spermatozoa

RAQUEL TREJO[1] AND ADELA MUJICA[2]
[1]*Sec. Regulación Metabólica, Unidad de Investigación Biomédica, Instituto Mexicano del Seguro Social;*
[2]*Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico*

ABSTRACT Calmodulin has been postulated as a mediator in the calcium-dependent processes that culminate in the acrosome reaction. Changes in calmodulin compartmentalization as a consequence of the increased permeability to extracellular calcium during capacitation and acrosome reaction have been suggested. In the present study the temporal localization of calmodulin in guinea pig spermatozoa was studied during in vitro capacitation and acrosome reaction by indirect immunofluorescence. Capacitation was achieved by incubation in Tyrode medium supplemented with pyruvate, lactate, and glucose in the presence and in the absence of calcium. Acrosome reaction was elicited in three different conditions: 1) by transfer to minimal culture medium containing pyruvate and lactate (MCM-PL) after in vitro capacitation, 2) by 0.003% Triton-X 100 treatment, and 3) by A 23187 addition to sperm samples incubated in MCM-PL. During capacitation, calmodulin was observed both in the acrosome and in the flagellum; this localization seemed to be independent of the presence of extracellular calcium and of exogenous substrates. Throughout the acrosome reaction, different stages of calmodulin compartmentalization were observed. It became clustered around the equatorial region just before or a little after the acrosome reaction had occurred. Later, it was observed around the postacrosomal region in the acrosome-reacted sperm. The changes in calmodulin distribution were found to be dependent on the stage in the acrosome reaction.

Key Words: In vitro capacitation, Sperm domains, Calcium

INTRODUCTION

Different cell surface domains have been detected in mammalian spermatozoa by freeze-fracture studies (Friend, 1982), immunofluorescence labelling (Gaunt et al., 1983; Myles et al., 1981; Primakoff and Myles, 1983), lectin binding (Koehler, 1978; Nicolson and Yanagimachi, 1974; Nicolson et al., 1977), and probes for anionic lipids (Bearer and Friend, 1980, 1982). Although no major morphological changes have been observed (Bedford, 1969), removal of surface components (Eng and Oliphant, 1978; Rufo et al., 1982) and migration and redistribution of plasma membrane proteins have been reported as occurring during the capacitation of spermatozoa from different species (Saxena et al., 1986a,c; Feuchter et al., 1986; Langlais and Roberts, 1985). The acquisition of an increased permeability to extracellular calcium is a major consequence of these biophysical and biochemical changes in the sperm plasma membrane. Capacitation has been postulated as a reversible phenomenon until the intracellular concentration of calcium reaches the threshold level required to activate the process of acrosome reaction. Different approaches have been developed to define whether calmodulin as a primary calcium-binding protein (Means and Dedman, 1980; Cheung, 1980) participates in the acrosome reaction.

Phenothiazines (Levin and Weiss, 1979) and naphthalene sulfonamides (Hidaka et al., 1979, 1980) have been employed to test the functioning of calmodulin, but the effects of these drugs on the acrosome reaction were too complex to be attributable exclusively to calmodulin (Lenz and Cormier, 1982; Nagae and Srivastava, 1986; Umekawa et al., 1985; Tanaka et al., 1985, Van Belle, 1984). Calmodulin distribution both in the whole cell and in subcellular fractions has been determined with immunocytochemical techniques (Olson et al., 1985; Noland et al., 1985; Wasco and Orr, 1984). In these experiments, calmodulin was seen to be

Received August 7, 1989; accepted March 13, 1990.
Address reprint requests to Dr. Adela Mújica, Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN, Apdo. Postal 14-740, 07000 Mexico D.F., Mexico.
Raquel Trejo's present address is Unidad de Investigación Clínica en Enfermedades Oncológicas, Subjefatura de Investigación, Apdo Postal 73032, Mexico D.F., Mexico.

associated with defined regions of the head and to specific parts of the flagellum (Jones et al., 1980; Feinberg et al., 1981; Gordon et al., 1983; Moore and Dedman, 1984; Camatini et al., 1986). It was suggested that this restricted localization of calmodulin might be related to intracellular calcium concentration gradients (Camatini et al., 1986a; Olson et al., 1985).

After due consideration of these different observations, we formulated the hypothesis that calmodulin distribution in spermatozoa might represent transient stages of association that evolved as a result of an increased calcium influx during the course of capacitation and the acrosome reaction (Singh et al., 1978). The first step in pursuing this hypothesis was to study the temporal sequence of calmodulin localization throughout in vitro capacitation and during the acrosome reaction. Guinea pig spermatozoa were incubated in two chemically defined capacitating media: Tyrode's medium plus pyruvate, lactate, and glucose (T-PLG) (Mújica and Valdés Ruiz, 1983) and minimal culture medium plus pyruvate and lactate (MCM-PL) (Rogers and Yanagimachi, 1975; Huang et al., 1981; Coronel and Lardy, 1987). Acrosome reaction was elicited in three different conditions: 1) by transfer to MCM-PL after in vitro capacitation of sperm in T-PLG medium (Mújica and Valdés Ruiz, 1983), 2) by treatment with 0.003% Triton-X 100 (Singh et al., 1978); and 3) by addition of A 23187 (Talbot et al., 1976) to sperm samples incubated in MCM-PL.

Our results suggested that calmodulin was preferentially associated with the acrosome and with the flagellum structures in T-PLG-capacitated spermatozoa. During the process of the acrosome reaction, calmodulin became compartmentalized to the equatorial and postacrosomal regions of the sperm head. This pattern of localization was obtained in the three experimental conditions tested.

MATERIALS AND METHODS

Chemicals

Analytical-grade chemicals were utilized. Sodium pyruvate, lactic acid, and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO); Triton X-100 was from BDH Chemicals Ltd.; Nembutal Anestesal was from Smith Kline Norden de México; D-glucose was from J.T. Baker (Xalostoc, Mexico); A 23187 was from Lilly Laboratories; and fluorescein-conjugated rabbit anti-sheep IgG was from Cappel Organon Teknica Laboratories.

Source of Proteins and Antibodies

Calmodulin was purified from bovine testicular tissue as Dedman and Kaetzel (1983) reported by affinity chromatography on phenol sepharose as Gopalakrishna and Anderson (1982) described. Polyacrylamide gel electrophoresis in the presence and in the absence of $CaCl_2$ and the absorbance spectrum in the ultraviolet region were used as criteria for calmodulin purity.

Polyclonal antibodies against calmodulin were prepared in sheep following a common immunization program and were purified by affinity chromatography on a calmodulin-sepharose column as Dedman et al. (1978) recommended. Antibodies titer, anticalmodulin, and fluorescein-conjugated rabbit anti-sheep IgG were determined by enzyme-linked immunosorbent assay (ELISA).

Incubation Media

T-PLG, pH 7.6, was prepared according to Rogers and Yanagimachi (1975) as modified by Mújica and Valdés Ruiz (1983). In some experiments, $CaCl_2$ was omitted. MCM-PL, pH 7.8 (Rogers and Yanagimachi, 1975), and 154 mM NaCl were used. All the solutions and media employed were fresh and had been filtered through 0.45 μm Millipore filters before use.

Sperm Preparation and Incubation Procedure

Spermatozoa were obtained from the vasa deferentia of two to five adult guinea pigs anaesthetized with Nembutal (50 mg/kg) and killed by cervical dislocation. Dissected ductus were perfused with 154 mM NaCl (2 ml/duct) at 37°C; immotile or contaminated samples were discarded. Pooled spermatozoa were washed twice with half the original volume of 154 mM NaCl and spun at 600*g* 4 min. Sperm concentration was determined during the second saline wash; 50 μl aliquots of the sperm suspension were diluted to 1 ml with 0.1 Triton-X 100 and counted in a Newbawer chamber.

Spermatozoa pellets were resuspended in the different media to a final concentration of 35×10^6 cells/ml and incubated under air atmosphere at 37°C for the appropriate time. It was variable for each experimental condition (see text). Throughout the incubation, aliquots of sperm suspensions were examined by light microscopy for motility and acrosome reaction occurrence.

A23187 sperm treatment. Ten microliters of 19 μM A 23187 in DMSO was added per milliliter of MCM-PL sperm suspension. The final concentration was 190 nM and 1% for A 23187 and DMSO, respectively.

Detergent sperm treatment. Triton-X 100 at 0.003% (w/v) final concentration in MCM-PL was used to resuspend and to incubate the sperm pellet after the saline washes.

Estimation of the Acrosome Loss

The percentage of spermatozoa without acrosome was determined in sperm samples fixed in 3% formaldehyde-DPBS (136 mM NaCl; 2.6 mM KCl; 1.4 mM KH_2PO_4; 8 mM Na_2HPO_4; 0.49 mM $MgCl_2$; 0.68 mM $CaCl_2$, pH 7.2). At least 300 cells were counted in duplicate samples.

Indirect Immunofluorescence Procedure

Sperm suspension aliquots were fixed in 3% formaldehyde-DPBS for 90 min at room temperature. At the end of this period, the fixative was removed and the cells were washed four times with 4 ml of DPBS and were incubated in 0.05 M NH_4Cl for 10 min and rinsed with DPBS. When sperm cells were resuspended in DPBS and smeared on microcoverslides (11 × 22 mm), the salt crystals that had formed after air-drying had a negative effect on spermatozoa distribution and morphology. A final distilled water wash was then included. Spermatozoa resuspended in distilled water were used to prepare the smears. After being air-dried at room temperature and being given a DPBS rinse, sperm were permeabilized in absolute acetone at −20°C for 7 min. DPBS was then used to eliminate the solvent. Twenty-five microliters of anticalmodulin antibody (O.D. = 0.3) were added to each microcoverslide following the procedure developed by Dedman et al. (1978) and were incubated at room temperature for 60 min. Exhaustive DPBS washed cells were incubated with 25 μl of fluorescein-labelled rabbit anti-sheep IgG diluted with DPBS 1:15 at room temperature and in the dark. Glycerol-DPBS, 9:1, pH 8, and nail polish were used to mount and seal the preparations. Cells were viewed through an American Optical H 120 microscope adapted for epi-illumination. Images were recorded on Kodak Tri-X film 400 ASA.

RESULTS

Calmodulin Localization in Sperm During In Vitro Capacitation

Sperm samples were incubated at 37°C under several conditions: in Tyrode plus calcium (T-PGL + Ca^{2+}), Tyrode without calcium (T-PLG − Ca^{2+}), MCM-PL, and 154 mM NaCl. To define the moment when spermatozoa reached the capacitated stage, aliquots of the sperm suspensions were transferred to fresh MCM-PL to test whether sperm were able to express the acrosome reaction in a synchronized manner a few minutes after transfer. Between 70% and 80% of the guinea pig spermatozoa population incubated in T-PLG + Ca^{2+} were able to show the acrosome reaction simultaneously in the first 15 min after transfer to MCM-PL. A similar percentage of acrosome reacted sperm was obtained after in vitro capacitation in T-PLG − Ca^{2+}.

In MCM-PL, spermatozoa required 60–90 min of incubation before the acrosome reaction could be observed. Under these conditions the number of acrosome-reacted sperm increased gradually, and the maximum percentage was observed after 2–3 hr of incubation.

Aliquots of sperm suspension incubated up to 72 min in 154 mM NaCl without calcium showed a 6% increase in acrosome loss within the first 15 min of transfer to MCM-PL. NaCl-incubated spermatozoa were therefore considered to be noncapacitated control sperm, but exclusively for this range of time.

Calmodulin immunolocalization was observed in sperm samples fixed during the incubation in the four media. It was found that with an incubation period of up to 30 min more than 90% of the examined cells presented a uniform pale fluorescence in the entire cell surface (Fig. 1A). A very small number of cells, fewer than 4%, presented a slightly bright equatorial band. Almost the entire population presented its acrosome intact.

Between 30 and 72 min of incubation in T-PLG − Ca^{2+}, and in NaCl, acrosome loss was observed in 12%. This proportion increased to 23% in T-PLG + Ca^{2+} and in MCM-PL media. Most of these cells showed a fluorescent equatorial belt. The fluorescence of the flagellum seemed to increase in the middle piece after 72 min of incubation in T-PLG + Ca^{2+} or in T-PLG − Ca^{2+} (Fig. 1B).

Calmodulin Localization in Sperm Throughout the Acrosome Reaction

Synchronized acrosome reaction. Samples of T-PLG-capacitated spermatozoa transferred to MCM-PL were fixed at several time intervals, and calmodulin distribution was studied by immunofluorescence. Decoration patterns were defined after counting at least 400 cells from two different experiments at each of the intervals of time selected (Table 1).

From 30 sec to 3 min in MCM-PL, about 80% of sperm cells showed intact acrosome (Table 1a,b), surrounded by fine brilliant points, sometimes more evident at the apical surface. Fewer than 5% presented a fluorescent ring on the lower one-third of the head and a faint acrosome (Table 1c). The flagellum was brighter in the middle piece. Spermatozoa without acrosome represented 5–8% of the total (Table 1d–f); most of them were decorated with an equatorial fluorescent belt (Table 1d).

At 5 min, 48% of sperm population showed no acrosome and presented several decoration patterns. The most commonly observed was a lack of fluorescence in the head, accompanied by bright flagellum (Table 1e). Spermatozoa without acrosome but with equatorial decoration increased to 11%. In 3% of acrosome-reacted sperm, a different calmodulin distribution was observable, and the immunofluorescence was localized in the postacrosomal region (Table 1f).

Between 10 and 15 min after transfer to MCM-PL acrosome-reacted sperm rose to over 90%. The majority, 80%, did not show fluorescence in the head, but the tail was clearly brilliant (Table 1e, Fig. 4B). The number of spermatozoa decorated in the equatorial region did not change. Over longer periods of incubation in MCM-PL, the percentage of acrosome-reacted sperm decorated with a continuous fluorescence from the postacrosomal region to the attachment of the flagellum increased from 3% to 95% (Table 1f, Fig. 4c).

The comparative analysis of the immunofluorescence localization of calmodulin and acrosome reaction

TABLE 1 CALMODULIN LOCALIZATION THROUGHOUT SYNCHRONOUS ACROSOME REACTION ELICITED BY TRANSFER TO MCM-PL. IMMUNOFLUORESCENCE PATTERNS (%)

Time after Transfer to MCM-PL min	 a	b	c	d	e	f
0.5	79.4	4.2	3.0	8.0	0.3	0.3
1	79.7	12.4	2.6	4.6	0.1	0.5
3	66.0	19.6	4.4	8.0	0.4	1.0
5	36.8	11.6	3.8	11.0	34	3.2
10	5.4	0.22	2.9	10.4	79.8	1.1
15	3.8	--	3.1	11.0	80	2.0
30	0	2.5	0.4	1.5	--	95.6

Washed guinea pig spermatozoa were capacitated in T-PLG medium without calcium by incubation at 37°C for 72 min. The cells were collected by centrifugation, resuspended in MCM-PL medium, and returned to the bath (37°C) for acrosome reaction to be expressed. Aliquot samples were formaldehyde fixed at the time intervals indicated. Sperm calmodulin distribution was studied with indirect immunofluorescence. From 400 to 1,000 cells were assessed at each of the intervals of time selected.

occurrence in samples of noncapacitated spermatozoa incubated in 154 mM NaCl after transfer to MCM-PL helped us to identify those calmodulin binding sites probably not dependent on the expression of the acrosome reaction (Table 2). From 30 sec to 15 min after transfer to MCM-PL, 90% of the NaCl incubated spermatozoa showed acrosome and a decoration restricted to the middle piece of the tail; the rest of the cell, the acrosome and the principal piece, did not present fluorescence (Table 2a). Only 2% exhibited an equatorial fluorescent band (Table 2c). The proportion of spermatozoa with brilliant acrosome and postacrosomal decoration was under 1% (Table 2b). Spermatozoa without acrosome represented about 5%; most of them showed equatorial decoration and only 1% presented a brilliant postacrosomal fluorescence (Table 2d,f, respectively). In order to define whether the localization of calmodulin was dependent or not on the conditions necessary to elicit the acrosome reaction, two different methods to accelerate this process were chosen: treatment with 0.003% Triton-X 100 and addition of the calcium ionophore A 23187.

Accelerated acrosome reaction by Triton-X 100. Recently prepared guinea pig spermatozoa were incubated in MCM-PL and in MCM-PL + 0.003% Triton-X 100 (w/v) as per Singh et al. (1978). Aliquots of the two sperm suspensions were observed under light microscopy to define the acrosome reaction occurrence and the motility quality. Figure 2 shows the results obtained in two different experiments. The acrosome reaction in detergent-treated spermatozoa was accelerated in both cases. The maximum percentage of acrosome-reacted sperm in 0.003% Triton-X 100 + MCM-PL was reached 70–90 min earlier, and the motility was similar to or even better than that in control MCM-PL-incubated sperm.

Samples of spermatozoa were fixed at the times of maximum acrosome reaction to study calmodulin distribution; at 120 and 160 min for control; and at 50, 60, and 105 min for detergent-treated sperm. It was found

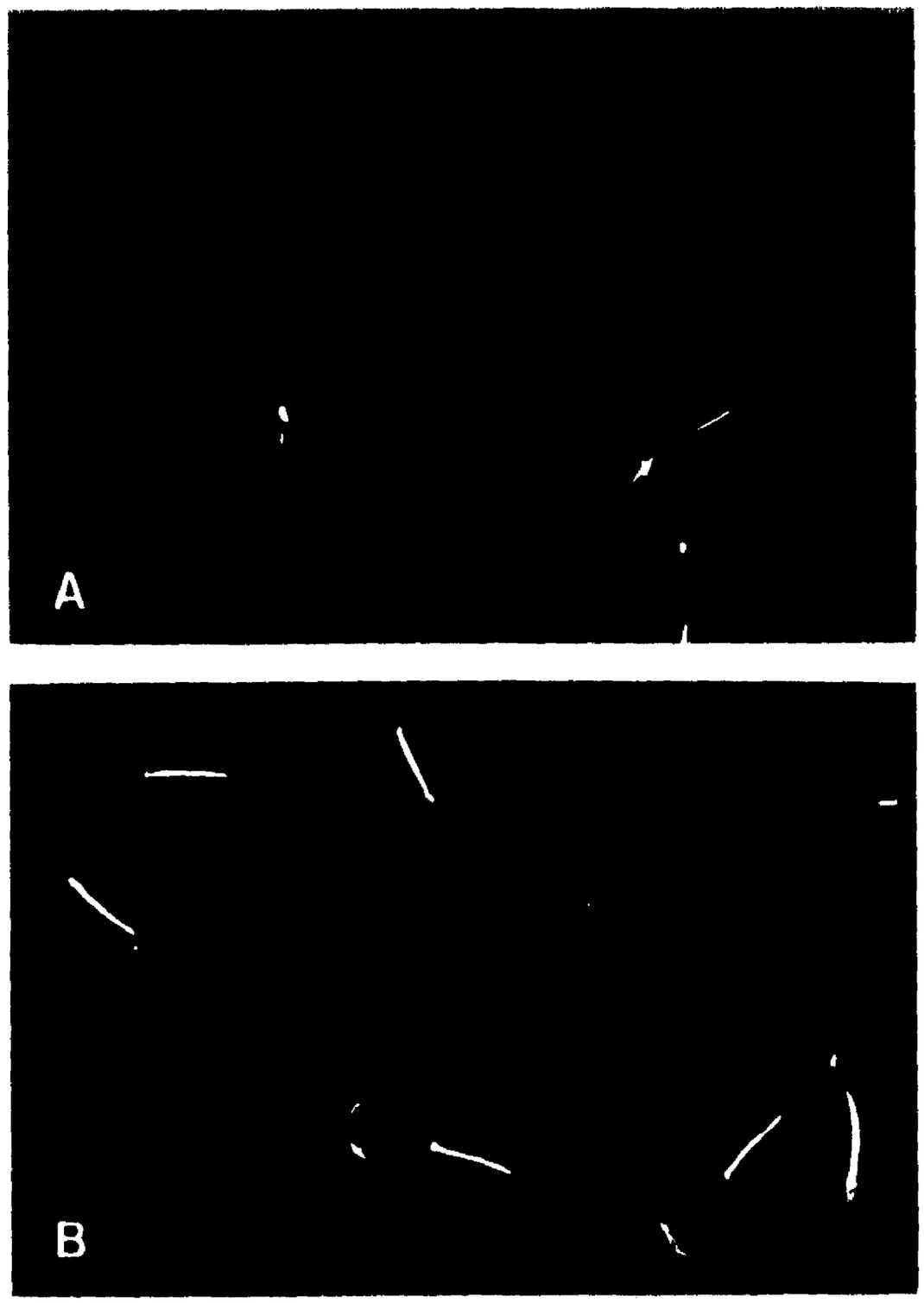


Fig. 1. Indirect immunofluorescence localization of calmodulin in guinea pig spermatozoa throughout capacitation. **A:** Partially or non-capacitated, intact sperm cells showed uniform pale fluorescence on the acrosomal cap and along the flagella (arrowhead). From 30 min, sperm incubation in 154 mM NaCl, T-PLG (+ calcium) or MCM-PL. **B:** Capacitated spermatozoa; the fluorescence of the flagellum seemed to increase in the middle piece. Sperm incubation after 72 min in T-PLG with or without calcium. × 1,100.

that, after 50–60 min of incubation, exclusively in Triton-X 100 + MCM-PL, numerous fluorescent vesicles accompanied the acrosome-reacted sperm. It was not possible to define whether these vesicles represented aggregates of acrosomal content or fragments of disrupted acrosome. This material was not observed in samples obtained at 105 min of incubation.

The head of the acrosome-reacted sperm did not show immunofluorescent labelling, but its flagellum was bright. This pattern was observed in control MCM-PL and in Triton-X 100-incubated spermatozoa. This decoration seemed to be equivalent to the one previously observed during the synchronous acrosome reaction of T-PLG + Ca^{2+} capacitated spermatozoa after transfer to MCM-PL (Table 1e).

Another experiment showed that the postacrosomal decoration may be observed if the incubation in MCM-PL continues 15–30 min after the acrosome reaction has taken place. In the case of spermatozoa incubated in 0.003% Triton-X 100 + MCM-PL, the localization of immunofluorescence in the equatorial region was evident only in samples fixed before the maximum percentage of acrosome reaction was reached.

A 23187-elicited acrosome reaction. MCM-PL supplemented with calcium ionophore A 23187, dissolved in DMSO, was used to induce the acrosome re-

TABLE 2 CALMODULIN LOCALIZATION IN NONCAPACITATED CONTROL SPERMATOZOA AFTER TRANSFER TO MCM-PL. IMMUNOFLUORESCENCE PATTERNS (%)

Time after Transfer to MCM-PL min	a	b	c	d	e	f
0.5	96.2	0.1	1.0	1.8	0.5	0.2
1	98.0	0.2	0.3	1.2	–	0.2
5	96.4	0.4	1.3	1.7	–	0.2
10	95.7	–	1.3	1.7	1.0	0.2
15	91.8	–	1.5	3.4	2.3	1.0

Washed guinea pig spermatozoa were incubated in 154 mM NaCl solution at 37°C for up to 72 min. Cells were incubated and then collected by centrifugation, resuspended in MCM-PL medium, and held at 37°C. Sperm suspension aliquots were fixed in formaldehyde at the indicated time intervals. Calmodulin distribution pattern on sperm cells was assessed by indirect immunofluorescence. At each time, more than 400 cells were observed to determine the percentage for each figure.

action in noncapacitated guinea pig spermatozoa. Three different concentrations of the ionophore were tested. At a final concentration of 190 nM, equivalent to 5.42 pmoles/10^6 spermatozoa and to a ratio of 422 pmoles/mg of protein, the ionophore accelerated the acrosome loss (Fig. 3A), reaching the highest value at 6 min after being added, but without activation of sperm motility. The examination of the sperm samples by light microscopy showed that the motility of the ionophore-treated sperm was similar to the motility of control samples incubated in MCM-PL alone or in MCM-PL + DMSO. A true acrosome reaction, involving both activation of motility with a whiplash flagellar movement and acrosome loss, occurred simultaneously at 40 min of incubation in A 23187-treated sperm and in the two control samples. Spermatozoa with intact acrosome after this treatment showed a pale fluorescence in the acrosome, in the equatorial region, and in the middle piece of the tail.

Sperm samples incubated for 20 min in MCM-PL were treated with A 23187 at a final concentration of 1.225 μM, equivalent to 35 pmoles/10^6 and to 2,725 pmoles/mg of protein. Aliquots of this suspension were fixed from 10 sec to 7 min after ionophore addition to determine the percentage of acrosome loss and the immunofluorescence localization of calmodulin. Figure 3B shows that the acrosome-reacted sperm gradually increased after a period of 1–4 min and the maximum percentage of acrosome reaction was observed at 6 min. Immunofluorescence of calmodulin in these samples is shown in Table 3. At 10 sec, 85% of the sperm population presented intact fluorescent acrosome and a brilliant middle piece (Table 3a–c). In acrosome-reacted sperm, a new pattern of decoration was observed. Brilliant points localized in the region previously occupied by the acrosome were observed in practically all the spermatozoa that were examined (Table 3e–g). Only 6% presented equatorial decoration (Table 3d,g). The frequency of this pattern increased in sperm samples incubated for a period of up to 7 min in the presence of the ionophore. The maximum value was reached at 7 min, when it represented 77% of the total acrosome-reacted sperm (Table 3d,e,g, Fig. 4A).

Other important findings included changes in the frequency of equatorial and postacrosomal decoration. The fluorescent equatorial band in sperm with intact acrosome (Table 3c) showed an average value of 35% from 10 sec to 2 min, which decreased to 21% at 4 min

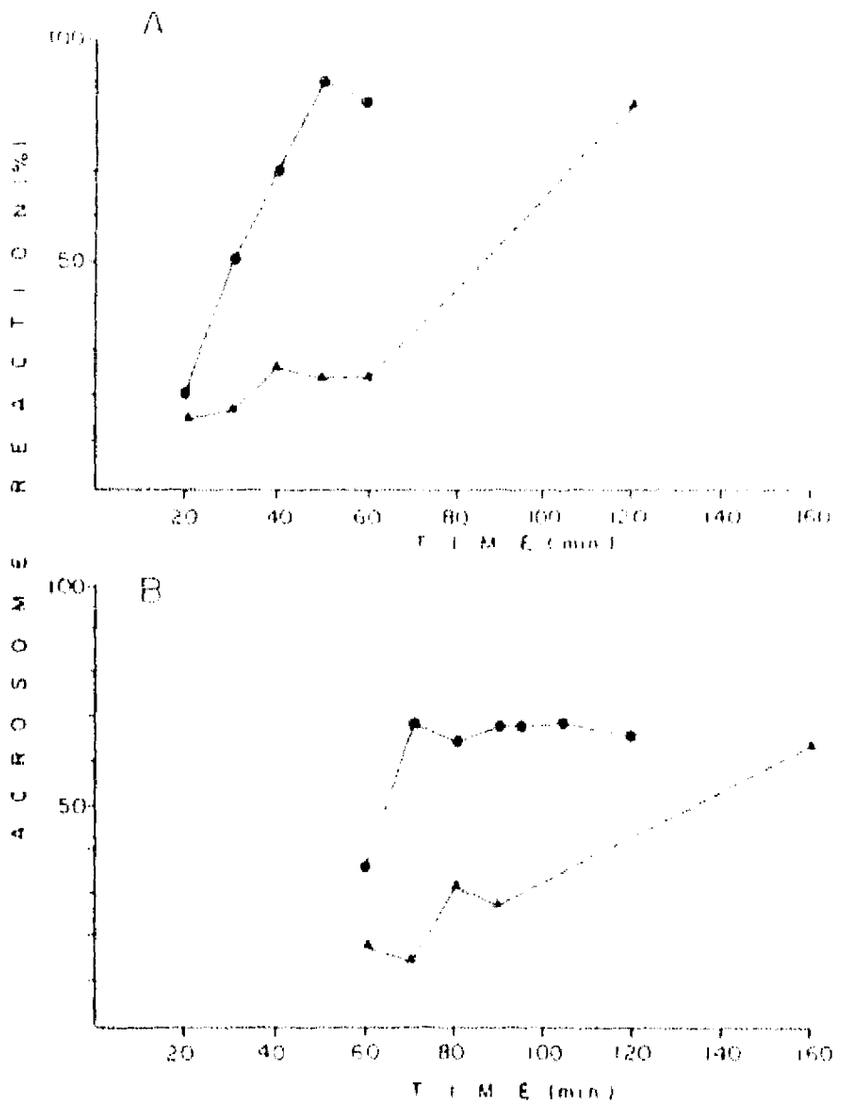


Fig. 2. Accelerated acrosome reaction by Triton X-100. Washed guinea pig spermatozoa were incubated in MCM-PL (▲) or MCM-PL plus 0.003% Triton X-100 (●). **A, B:** Two separate experiments. Once spontaneous acrosome reaction started in either sample incubation, aliquots were formaldehyde-fixed to assess the acrosome reaction.

and to 15% at 7 min. The presence of postacrosomal decoration was found to be more related to the spermatozoa without acrosome: a gradual increase from 8% at 10 sec to 34% at 4 min was observed (Table 3e,f). This decoration represented only 3% in sperm with intact acrosome (Table 3b).

At higher concentrations of ionophore, 2.45 μM, equivalent to 70 pmoles/10^6 sperm and to 5,444 pmoles/mg of protein, a complete blockage of any type of flagellar motion was observed. This inhibitory effect on sperm motility was also observed after 8 min on incubation in the presence of 1.336 μM A 23187. Figure 4 shows the major sperm calmodulin immunofluorescence patterns observed throughout the acrosome reaction.

DISCUSSION

The localization of calmodulin in the acrosome and along the tail in guinea pig spermatozoa during in vitro capacitation seemed to be independent of both extracellular calcium and exogenous substrates. The decoration pattern obtained in the four experimental conditions tested 154 mM NaCl, T-PLG − Ca^{2+}, T-PLG + Ca^{2+}, and MCM-PL, did not show any differences (Fig. 1). Calcium permeability in guinea pig sperm has been found to be increased by incubation in MCM-PL (Singh et al., 1978). Capacitation was also achieved by brief time incubation in T-PLG medium (Mújica and Valdés Ruiz, 1983). The presence or absence of calcium during capacitation affects neither the ^{45}Ca uptake (Singh et al., 1978) nor the time of incubation required for the acrosome reaction to take place (Yanagimachi and Usui, 1974; Mújica and Valdés Ruiz, 1983).

Singh et al. (1978) and Coronel and Lardy (1987),

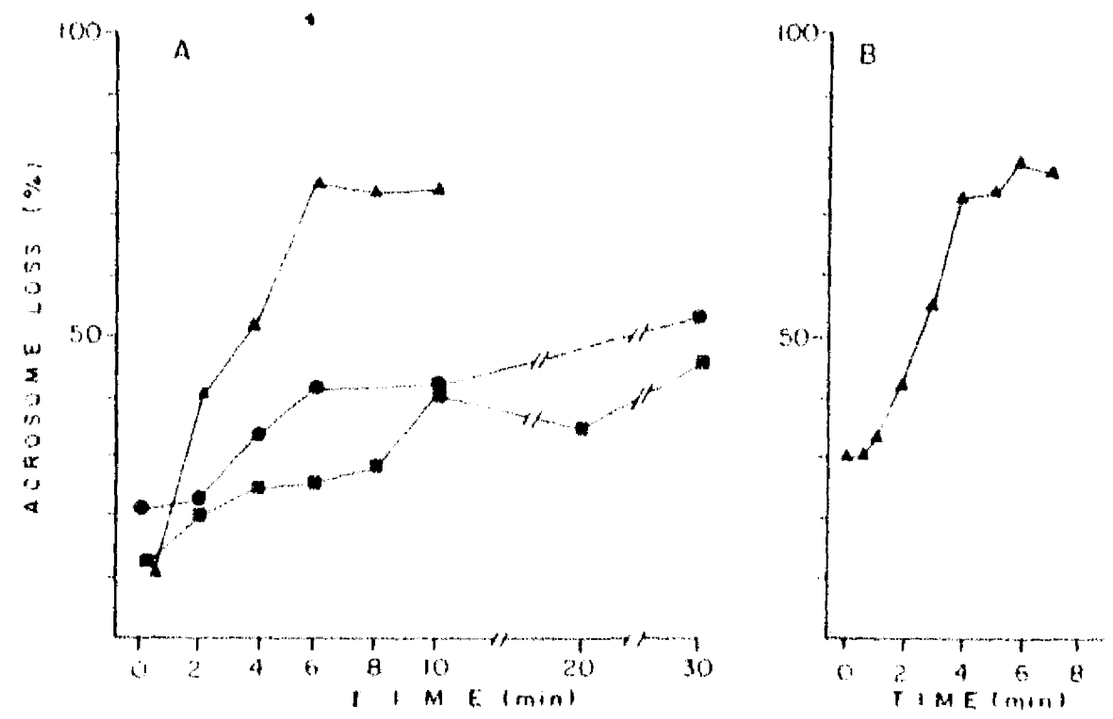


Fig. 3. A 23187-elicited acrosome reaction. **A:** Spermatozoa were incubated in MCM-PL (●) and in this medium supplemented with DMSO (●) or calcium ionophore A 23187 (▲) in DMSO (A 23187, 5.42 pmoles/10^6 spermatozoa·ml). **B:** Spermatozoa were preincubated for 20 min in MCM-PL medium before A 23187 was added (35 pmoles/10^6 sperm/ml). The percentage of acrosome-reacted sperm was assessed in formaldehyde-fixed aliquots withdrawn at the indicated times.

TABLE 3. CALMODULIN LOCALIZATION DURING A23187-INDUCED ACROSOME REACTION IMMUNOFLUORESCENCE PATTERNS (%)

Time after A 23187 Addition min	a	b	c	d	e	f	g
0.16	47.69	3.2	35.32	0.45	—	7.79	5.5
0.5	42.43	0.5	38.26	7.14	—	4.59	7.14
1	40.48	—	31.77	4.04	3.23	14.77	6.47
2	25.30	1.2	38.15	2.4	0.8	12.54	17.67
4	9.08	2.13	21.39	2.67	2.13	31.54	33.68
7	4.06	1.52	14.72	9.64	1.52	18.2	50.25

Spermatozoa were incubated for 20 min in MCM-PL, then supplemented with calcium ionophore A 23187 at 35 pmoles/10^6 sperm/ml. Aliquots were fixed after ionophore addition. Immunofluorescence localization of calmodulin in at least 200 cells was evaluated at each of the intervals of indicated time. The percentage of each distribution calmodulin pattern is indicated.

when characterizing the calcium uptake in guinea pig spermatozoa during in vitro capacitation, identified an initial calcium accumulation that was probably due to calcium-binding to the sperm surface, which was insensitive to mitochondrial inhibitors. The secondary phase of calcium-uptake was seen to be dependent on exogeneous substrates and selectively abolished by inhibitors and uncouplers of oxidative phosphorylation.

It is possible that, during capacitation, and in the absence of increased intracellular calcium levels, calmodulin becomes associated with specific regions of guinea pig sperm. This interpretation is supported by the following observations: the presence of several calmodulin binding proteins both in the outer acrosomal membrane complex (Olson et al., 1985) and in the plasma membrane (Noland et al., 1985) of bovine spermatozoa; the ability of these proteins to bind calmodulin in the presence of EGTA but not in the presence of calcium; and the lack of change in the cytoplasmic concentration of free calcium after in vitro capacitation in ejaculated rabbit spermatozoa (Mahanes et al., 1986).

The synchronous progression of the acrosome reaction in T-PLG – Ca^{2+}-capacitated sperm after transfer to MCM-PL allowed us to sequence the localization of calmodulin throughout this process. The clustering of calmodulin in the equatorial region was a transient stage appearing just prior to and lasting few minutes after the acrosome reaction, possibly linked to the secondary phase of calcium uptake. This stage was better observed in A 23187-treated spermatozoa, probably because the ionophore synchronizes more efficiently the whole sperm population at the stages of calcium entry required for the acrosome reaction. It was suggested that the ionophore enables the calcium ions to be transported to the site of action that is not accessible under physiological conditions until capacitation has taken place (Babcock et al., 1976, 1978; Coronel and Lardy, 1987). The distribution of calcium after A 23187 treatment of spermatozoa was found to be nonrandom. During the earliest stages of vesiculation, calcium was concentrated in the acrosome region just in front of the equatorial segment (Watson and Plummer, 1986; Plummer and Watson, 1985).

The absence of decoration in the nuclear region of the

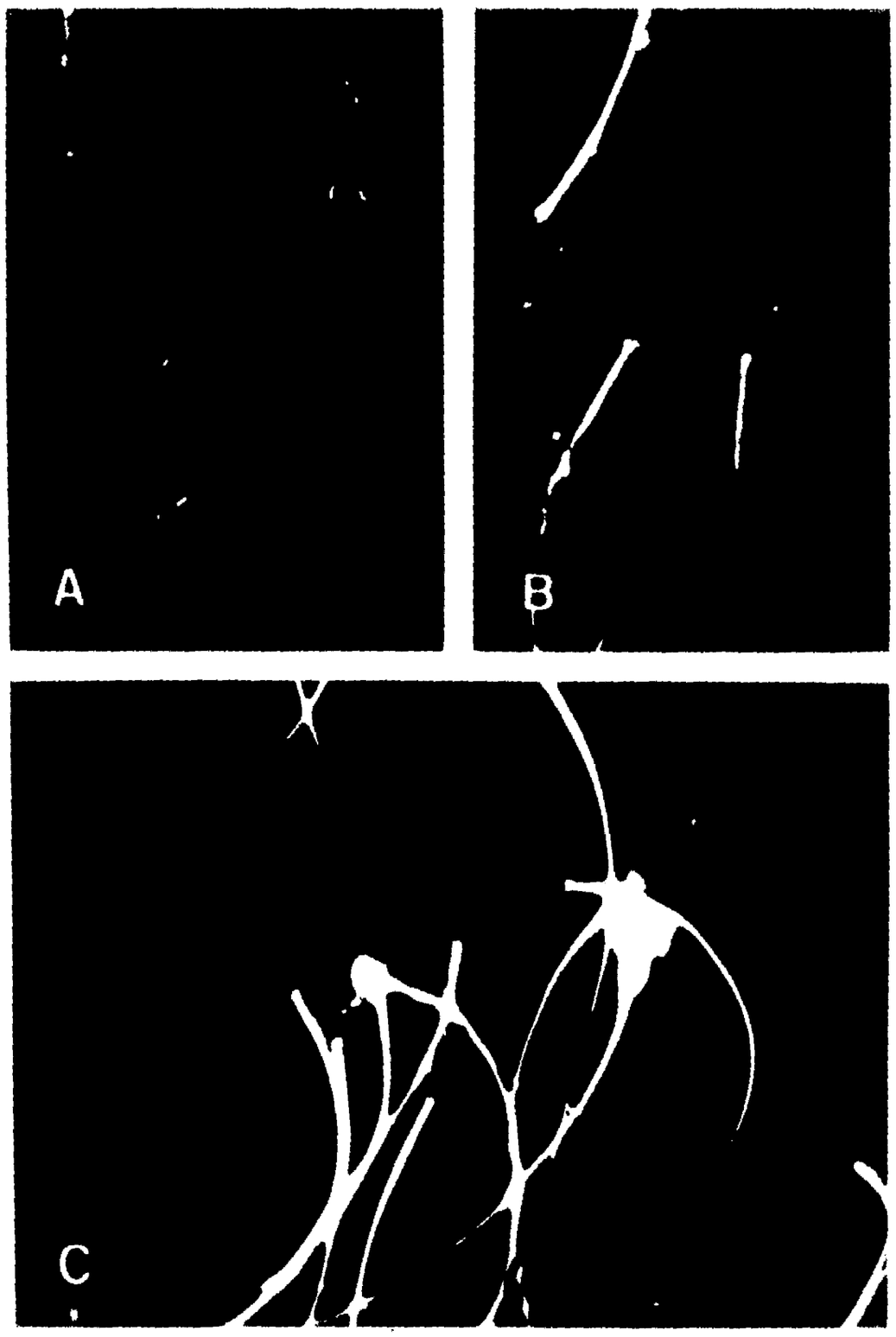


Fig. 4. Major sperm calmodulin immunofluorescence patterns, throughout acrosome reaction. A: Equatorial decoration in sperm without acrosome from A 23187-treated spermatozoa. B: Absence of decoration in the nuclear region. This pattern is observed a few minutes after the acrosome reaction has taken place by transferring capacitated sperm to MCM-PL medium. C: Localization of calmodulin to the postacrosomal region. This calmodulin distribution is present in sperm after 15–30 min acrosome reaction occurred, either by transfer of capacitated sperm to MCM-PL medium or in non-capacitated sperm by calcium ionophore (A 23187) treatment. × 1,100.

acrosome-reacted sperm was not a constant pattern. It was not observed in ionophore-treated spermatozoa. It remains to be seen whether the immunofluorescence in the region previously occupied by the acrosome reflects calmodulin binding sites attached to the inner acrosomal membrane or whether it is just an incomplete exocytosis of acrosomal content.

The localization of calmodulin immunofluorescence in the postacrosomal region of the head in acrosome-reacted sperm may be the result of modifications in spermatozoa domains. One possible explanation of this result is that calmodulin diffuses free from the flagellum domains and spreads to the postacrosomal region. It is very likely that the properties of this region change after the outer acrosomal membrane fuses with the plasma membrane. Spermatozoa incubated in the presence of 0.003% Triton-X 100 + MCM-PL did not show any postacrosomal immunofluorescence, probably due to a partial extraction of calmodulin-binding membrane proteins, although the equatorial segment, the postacrosomal sheath, and the middle piece of the tail have all been shown to be resistant to higher con-

centrations of detergent than those employed in this work (Vishwanath et al., 1986; Wooding, 1973).

Mobile and fixed proteins on the plasma membrane of mammalian spermatozoa have been observed. Lateral free diffusion within the plasma membrane overlying the posterior region of the tail has been detected in an integral membrane protein (Myles et al., 1984). Antibody-induced patching of two antigens originally localized in the entire plasma membrane of the flagellum (Gaunt et al., 1983) and clustering of a glycoprotein exclusively at the acrosome surface (O'Rand, 1977) have also been observed. The mobility of these proteins was restricted to their specific domains, and patching was not always induced in spite of the antibody specificity (Gaunt et al., 1983). It has been suggested that barriers to diffusion exist in mammalian spermatozoa (Cowan et al., 1987).

Cytoskeletal elements have been involved in the immobilization and regionalized distribution of membrane constituents (Golan and Veatch, 1980; Branton et al., 1981). The major role played by microfilaments in the migration of plasma membrane proteins from the head to the flagellum has been demonstrated (Saxena et al., 1986b,c). The highly polarized organization of actin, myosin, vimentin, and nonerythroid spectrin in spermatozoa suggested that different cytoskeletal-cell surface assemblies were involved in the regulation of the acrosome reaction and fertilization (Virtanen et al., 1984). Nonfilamentous actin and calmodulin were clearly observed in the equatorial segment of boar spermatozoa (Camatini et al., 1986a,b). The colocalization of actin and calmodulin suggests that the aggregation state of actin may be controlled by calmodulin either by decreasing the levels of free calcium by means of its calcium binding properties or through the activation of regulatory proteins of F actin (Dedman et al., 1979; Means and Dedman, 1980; Kakiuchi and Sobue, 1983).

The evolution of calmodulin compartmentalization throughout the acrosome reaction confirmed our hypothesis concerning transient calmodulin associations dependent on the stage reached in the acrosome reaction. However, the precise role of calmodulin in this process was not defined by these experiments. Further work is required to identify the proteins that can bind calmodulin in a calcium-dependent and -independent manner as well as to determine the lateral diffusion of calmodulin.

ACKNOWLEDGMENTS

We are grateful to Dr. J.R. Dedman for his advice and Dr. C. Argüello for providing immunofluorescence microscope facilities. This research was partially supported by Consejo Nacional de Ciencia y Tecnologia grants PCCBBNA-022570 and PCSABNA-022046 to R.T. and A.M. respectively.

REFERENCES

Babcock DF, First NF, Lardy H (1976): Action of ionophore A 23187 at the cellular level. J Biol Chem 251:3881–3886.

Babcock DF, Stamerjhon DM, Hutchinson T (1978): Calcium redistribution in individual cells correlated with ionophore action on motility. J Exp Zool 204:391–400.

Bearer EL, Friend DS (1980): Anionic lipid domains: Correlation with functional topography in a mammalian cell membrane. Proc Natl Acad Sci USA 77:6601–6605.

Bearer EL, Friend DS (1982): Modifications of anionic lipid domains preceding membrane fusion in guinea pig sperm. J Cell Biol 92: 604–615.

Bedford JM (1969): Morphological aspects of sperm capacitation in mammals. Adv Biosci 4:35–50.

Branton D, Cohen CM, Tyler J (1981): Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell 24:24–32.

Camatini M, Anelli G, Casale A (1986a): Immunocytochemical localization of calmodulin in intact and acrosome-reacted boar sperm. Eur J Cell Biol 41:89–96.

Camatini M, Anelli G, Casale A (1986b): Identification of actin in boar spermatids and spermatozoa by immunoelectron microscopy. Eur J Cell Biol 42:311–318.

Cheung WY (1980): Calmodulin plays a pivotal role in cellular regulation. Science 207:19–27.

Coronel CE, Lardy HA (1987): Characterization of Ca^{2+}-uptake by guinea pig epididymal spermatozoa. Biol Reprod 37:1097–1107.

Cowan AE, Myles DG, Koppel DE (1987): Lateral diffusion of the PH-20 protein on guinea pig sperm: Evidence that barriers to diffusion maintain plasma membrane domains in mammalian sperm. J Cell Biol 104:917–923.

Dedman JR, Brinkley BR, Means AR (1979): Regulation of microfilaments and microtubules by calcium and cyclic AMP. Adv Cyclic Nucleotide Res 11:131–174.

Dedman JR, Kaetzel MA (1983): Calmodulin purification and fluorescent labelling. In AR Means and BW O'Malley (eds): "Methods in Enzymology." New York: Academic Press, Vol 102, pp 1–12.

Dedman JR, Welsh MJ, Means AR (1978): Ca^{2+}-dependent regulator: Production and characterization of a monospecific antibody. J Biol Chem 253:7515–7521.

Eng LA, Oliphant G (1978): Rabbit sperm reversible decapacitation by membrane stabilization with a highly purified glycoprotein from seminal plasma. Biol Reprod 19:1083–1094.

Feinberg J, Weinman J, Weinman S, Walsh MP, Harricane MC, Gabrion J, Demaille JG (1981): Immunocytochemical and biochemical evidence for the presence of calmodulin in bull sperm flagellum. Biochim Biophys Acta 673:303–311.

Feuchter FA, Tabet A, Green M, Gresham D (1986): Exposure and translocation of a cytoskeleton-linked surface glycoprotein during capacitation and the acrosome reaction in human sperm. Anat Rec 214:37a.

Friend DS (1982): Plasma-membrane diversity in a highly polarized cell. J Cell Biol 93:243–249.

Gaunt SJ, Brown CR, Jones R (1983): Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. Exp Cell Res 144:275–284.

Golan DE, Veatch W (1980): Lateral mobility of band 3 in the human erythrocyte membrane studied by fluorescence photobleaching recovery: Evidence for control by cytoskeletal interactions. Proc Natl Acad Sci USA 77:2537–2541.

Gopalakrishna R, Anderson WB (1982): Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem Biophys Res Commun 104:830–836.

Gordon M, Morris EG, Young RJ (1983): The localization of Ca^{2+}-ATPase and Ca^{2+}-binding proteins in the flagellum of guinea pig sperm. Gamete Res 8:49–55.

Hidaka H, Yamaki T, Naka M, Tanaka T, Hayashi H, Kobayashi R (1980): Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. Mol Pharmacol 17:66–72.

Hidaka H, Yamaki T, Totsuka T, Asano M (1979): Selective inhibitors of Ca^{2+}-binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin interaction. Mol Pharmacol 15: 49–59.

Huang TTF, Fleming AD, Yanagimachi R (1981): Only acrosome-reacted spermatozoa can bind to and penetrate zona pellucida: A study using the guinea pig. J Exp Zool 217:287–290.

Jones HP, Lenz RW, Palevitz BA, Cormier MJ (1980): Calmodulin localization in mammalian spermatozoa. Proc Natl Acad Sci USA 77:2772–2776.

Kakiuchi S, Sobue K (1983): Control of the cytoskeleton by calmodulin and calmodulin-binding proteins. Trends Biochem Sci 8: 59–62.

Koehler JK (1978): The mammalian sperm surface: Studies with specific labeling techniques. Int Rev Cytol 54:73–108.

Langlais J, Roberts KD (1985): A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Res 12:183–224.

Lenz RW, Cormier MJ (1982): Effects of calmodulin-binding drugs on the guinea pig spermatozoon acrosome reaction and the use of these drugs as vaginal contraceptive agents in rabbits. Ann NY Acad Sci 383:85–97.

Levin RM, Weiss B (1979): Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. J Pharmacol Exp Ther 208:454–459.

Mahanes MS, Ochs DL, Eng LA (1986): Cell calcium of ejaculated rabbit spermatozoa before and following in vitro capacitation. Biochem Biophys Res Commun 134:664–670.

Means AR, Dedman JR (1980): Calmodulin, an intracellular calcium receptor. Nature 285:73–77.

Moore PB, Dedman JR (1984): Calmodulin, a calmodulin acceptor protein, and calcimedins: Unique antibody localizations in hamster sperm. J Cell Biochem 25:99–107.

Mújica A, Valdés Ruiz MA (1983): On the role of glucose in capacitation and acrosomal reaction of guinea pig sperm. Gamete Res 8: 335–344.

Myles DG, Primakoff P, Bellve AR (1981): Surface domains of the guinea pig sperm defined with monoclonal antibodies. Cell 23:433–439.

Myles DG, Primakoff P, Koppel DE (1984): A localized surface protein on guinea pig sperm exhibits free diffusion in its domain. J Cell Biol 98:1905–1909.

Nagae T, Srivastava PN (1986): Induction of the acrosome reaction in guinea pig spermatozoa by calmodulin antagonist W-7. Gamete Res 14:197–208.

Nicolson GL, Usui N, Yanagimachi R, Yanagimachi H, Smith JR (1977): Lectin binding sites on the plasma membranes of rabbit spermatozoa. J Cell Biol 74:950–962.

Nicolson GL, Yanagimachi R (1974): Mobility and the restriction of mobility of plasma membrane lectin-binding components. Science 184:1294–1296.

Noland TD, VanEldik LJ, Garbers DL, Burgess WH (1985): Distribution of calmodulin and calmodulin binding proteins in membranes from bovine epididymal spermatozoa. Gamete Res 11:297–303.

Olson GE, Winfrey VP, Garbers DL, Noland TD (1985): Isolation and characterization of macromolecular complex associated with the outer acrosomal membrane of bovine spermatozoa. Biol Reprod 33: 761–779.

O'Rand MG (1977): Restriction of a sperm surface antigen's mobility during capacitation. Dev Biol 55:260–270.

Plummer JM, Watson PF (1985): Ultrastructural localization of calcium ions in ram spermatozoa before and after cold shock as demonstrated by a pyroantimonate technique. J Reprod Fertil 75:255–263.

Primakoff P, Myles DG (1983): A map of the guinea pig sperm surface constructed with monoclonal antibodies. Dev Biol 98:417–428.

Rogers BJ, Yanagimachi R (1975): Retardation of guinea pig sperm acrosome reaction by glucose: The possible importance of pyruvate and lactate metabolism in capacitation and acrosome reaction. Biol Reprod 13:568–575.

Rufo GA Jr, Singh JP, Babcock DF, Lardy HA (1982): Purification and characterization of a calcium transport inhibitor protein from bovine seminal plasma. J Biol Chem 257:4627–4632.

Saxena N, Peterson RN, Saxena NK, Russell LD (1986a): Microfilaments appear in boar spermatozoa during capacitation in vitro. J Exp Zool 239:423–427.

Saxena N, Sharif S, Saxena NK, Russell LD, Peterson RN (1986b): Changes in plasma membrane (PM) organization during in vitro capacitation of boar spermatozoa detected by monoclonal antibodies (MCAs). Fed Proc 45:305.

Saxena N, Peterson RN, Sharif S, Saxena NK, Russell LD (1986c): Changes in the organization of surface antigens during in vitro capacitation of boar spermatozoa as detected by monoclonal antibodies. J Reprod Fertil 78:601–614.

Singh JP, Babcock DF, Lardy HA (1978): Increased calcium ion influx is a component of capacitation of spermatozoa. Biochem J 172: 549–556.

Talbot P, Summers RG, Hylander BL, Keough EM, Franklin LE (1976): The role of calcium in the acrosome reaction: An analysis using ionophore A 23187. J Exp Zool 198:383–392.

Tanaka T, Inagaki M, Ito M, Hidaka H (1985): Calmodulin antagonists and protein kinase C. In H Hidaka and DJ Hartshorne (eds): "Calmodulin Antagonists and Cellular Physiology." New York: Academic Press, pp 525–529.

Umekawa H, Gaka M, Inagaki M, Hidaka H (1985): Interaction of W-7, a calmodulin antagonist with another Ca^{2+}-binding protein. In H Hidaka and DJ Hartshorne (eds): "Calmodulin antagonists and cellular physiology." New York: Academic Press, pp 511–524.

Van Belle H (1984): The effect of drugs on calmodulin and its interaction with phosphodiesterase. Adv Cyclic Nucleotide Protein Phosphorylation Res 17:557–567.

Virtanen I, Badley RA, Paasivuo R, Lehto VP (1984): Distinct cytoskeletal domains revealed in sperm cells. J Cell Biol 99:1083–1091.

Vishwanath R, Swan MA, White IG (1986): Effect of Triton X-100 on ultrastructure, reactivation and motility characteristics of ram spermatozoa. Gamete Res 15:361–371.

Wasco WM, Orr GA (1984): Function of calmodulin in mammalian sperm: Presence of calmodulin-dependent cyclic nucleotide phosphodiesterase associated with demembranated rat caudal epididymal sperm. Biochem Biophys Res Commun 118:636–642.

Watson PF, Plummer JM (1986): Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa. J Exp Zool 238:113–118.

Wooding FBP (1973): The effect of Triton X-100 on the ultrastructure of ejaculated bovine sperm. J Ultrastruct Res 42:502–516.

Yanagimachi R, Usui N (1974): Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res 89:161–174.

TISSUE AND CELL 1994 26 (6) 849–856
© 1994 Longman Group Ltd

ENRIQUE OTHÓN HERNÁNDEZ*, RAQUEL TREJO†, ANA MARÍA ESPINOSA†, ARTURO GONZÁLEZ§ and ADELA MUJICA*

CALMODULIN BINDING PROTEINS IN THE MEMBRANE VESICLES RELEASED DURING THE ACROSOME REACTION AND IN THE PERINUCLEAR MATERIAL IN ISOLATED ACROSOME REACTED SPERM HEADS

Keywords: Acrosome reaction vesicles, calmodulin binding sperm membrane proteins, calcium-independent calmodulin-binding proteins, sperm membrane solubilization.

ABSTRACT. Calmodulin has been suggested as the Ca^{2+} mediator in diverse cellular functions *via* its interaction with a number of proteins in a calcium dependent manner. Its participation in the acrosome reaction has been suggested based on its localization in the acrosomic region, on the effects produced by calmodulin antagonists, and by the changes in calmodulin compartmentation observed to occur throughout guinea pig acrosome reaction. To define the role of calmodulin in the membrane fusion events that occur during the acrosome reaction, the identification of calmodulin-binding proteins, by the overlay technique with biotinylated or unmodified calmodulin, was made in the following sperm fractions: in the membrane vesicles released during the acrosome reaction, in the remaining perinuclear material of acrosome reacted sperm heads and in a total membrane fraction from intact spermatozoa. The membrane vesicles released after the acrosome reaction showed four major calmodulin binding proteins, M_rs 66, 95, 97 and 110 kDa. The perinuclear material showed a 31–34, 43 and 92 kDa calmodulin-binding polypeptides. The membrane fraction from intact sperm showed eleven calmodulin-binding proteins, M_rs between 14–110 kDa. Most of the binding proteins detected by this method corresponded to the class of calcium-independent calmodulin-binding proteins but proteins which only interacted with calmodulin in a calcium-inhibited mode were also observed. No calcium-dependent calmodulin-binding proteins were detected in any of the fractions studied. A possible role of these binding proteins in calmodulin compartmentation is discussed. The potential role of these binding proteins in membrane fusion and in membrane receptor localization in the postacrosomal region remain to be defined.

Introduction

Several lines of evidence suggest that calcium plays a major role in the processes that confer fertilizing ability to spermatozoa. The acrosome reaction, hyperactivated motility and sperm-egg fusion are Ca^{2+}-dependent functions (Yanagimachi and Usui, 1974; Fraser, 1977, 1987a, 1987b; Aitken *et al.*, 1984, 1988; Yanagimachi 1982, 1988). Even though calmodulin is the major Ca^{2+}-acceptor protein, identified both in the soluble (Jones *et al.*, 1978) and in sperm membrane fractions (Noland *et al.*, 1985; Olson *et al.*, 1985; Weinman *et al.*, 1986; Aitken *et al.*, 1988), the mechanism by which this protein might transduce the calcium signal into a specific response, such as the acrosome reaction, is still unclear. The inhibitory effect of cal-

* Departamento de Biología Celular and §Departamento de Patología Experimental, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Apartado Postal 14740.07000 México, D.F. México.
† Unidad de Investigación Clínica en Enfermedades Oncológicas, IMSS.
‡ Present address: División de Investigación Básica, Instituto Nacional de Cancerología.
Correspondence to: E. O. Hernández.

Received 15 December, 1993
Accepted 15 June, 1994

modulin antagonists such as phenothiazines (Levin and Weiss, 1979) and naphthalene sulfonamides (Hidaka *et al.*, 1979, 1980) on the acrosome reaction and on gamete fusion was considered as an indirect demonstration of calmodulin involvement in these processes (Lenz and Cormier, 1982; Sano, 1983; Aitken *et al.*, 1988; Lydan and O'Day, 1988). Although the interactions of these drugs with other proteins and with membrane lipids (Umekawa *et al.*, 1985; Tanaka *et al.*, 1985; Orrego *et al.*, 1985) offer alternative interpretations, the participation of calmodulin in membrane fusion events is also suggested by the changes in calmodulin localization observed throughout the acrosome reaction (Trejo and Mújica, 1990), by the redistribution of calmodulin, temporally correlated with the onset of myoblast fusion (Bar-Sagi and Prives, 1983), and by the changes in membrane fluidity after Ca^{++}-binding in the presence of calmodulin (De Lorenzo, 1980; Kopeikina-Tsibouridou and Deliconstantinos, 1986, 1989).

To understand the mechanism by which calmodulin may participate in the membrane fusion events that occur during the acrosome reaction, the isolation of the sperm membranes involved in this process and the identification of their calmodulin-binding proteins were undertaken in the present work. The hypothesis that specific calmodulin-binding proteins may be present in the acrosomal and postacrosomal regions which might participate in the fusion of the plasma and outer acrosomal membranes and in the postacrosomal localization of calmodulin in acrosome reacted spermatozoa was pursued in this work. We decided to study the following sperm membranes: (a) the plasma and acrosomal membrane fraction obtained from intact non-reacted spermatozoa, (b) the membrane vesicles produced by the fusion of the plasma membrane with the outer acrosomal membrane during the acrosome reaction, and (c) the perinuclear material obtained from isolated acrosome-reacted sperm heads. Biotinylated and unmodified bovine calmodulin were employed to overlay nitrocellulose blots of these fractions resolved by SDS-PAGE electrophoresis.

Only calcium-independent and calcium-inhibited calmodulin binding proteins were detected with both calmodulin probes. The similarity in molecular weight of some peptides from the three fractions studied suggests the presence of common modulator-binding proteins in these membranes. The lack of calcium-dependence to interact with calmodulin suggests that these proteins may play a role in calmodulin compartmentation.

Materials and Methods

Protein A-gold conjugate was from E.Y. Laboratories (San Mateo, Ca., USA). Rabbit anti-sheep IgG labeled with peroxidase or with fluorescein was from Cappel (Organon Teknika N.V., Belgium). Rabbit anti-goat IgG labeled with peroxidase was from HyClone (Logan, Utah, USA). Biotinylated calmodulin was from Gibco BRL (Gaithersburg, MD). Acrylamide, bisacrylamide, TEMED, molecular weight standards, and nitrocellulose membrane were from BioRad (Richmond, Ca., USA). Sodium dodecyl sulfate (SDS) and Triton X-100 were from BHD Chemical Ltd. (Poole, UK). Cyanogen bromide and dimethyl formamide were from Merck (Schuchardt, Germany). Acetonitrile and hydroquinone from J. T. Baker (USA). Phenyl-methane-sulfonyl fluoride (PMSF), p-hydroxy-mercuribenzoate (pHMB), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), leupeptin (acetyl-leu-leu-arg-al), p-aminobenzamidine (pABA), N-ethyl-maleimide (NEM), 3'3'-diaminobenzidine tetrahydrochloride (DAB), Tween 20, bovine serum albumin (BSA), ethylene-diamine-tetracetic acid, disodium salt (EDTA), silver lactate, ionophore A23187, trifluoroperazine (TFP), DL-dithiothreitol (DTT), lactic acid, o-phenylendiamine, cetyl-trimethyl-ammonium bromide (CTAB), and glycine were from Sigma Chemical Co. (St. Louis, Mo., USA).

Source of antibodies

Goat anti-calmodulin polyclonal antibody was from Sigma Chemical Co. (St. Louis, Mo., USA). Sheep anti-calmodulin polyclonal antibodies were raised using bovine calmodulin purified from testis as previously described (Gopalakrishna and Anderson, 1982; Dedman and Kaetzel, 1983). 10 mg of heated-calmodulin (3 min at 90°C) re-suspended in 1 ml of Freund's complete adjuvant were injected subcutaneously the first time. 5 mg of calmodulin re-suspended in Freund's incomplete adjuvant were admin-

istered the second and third times. Antibodies were purified by affinity chromatography on a calmodulin sepharose column as Dedman *et al.* (1978) recommended and titered by enzyme-linked immunoabsorbent assay (ELISA).

Spermatozoa isolation

Sperm cells were obtained from the vasa deferentia of adult guinea pigs anaesthetized with Nembutal (50–100 mg/kg) and killed by cervical dislocation. Dissected ductus were perfused with unbuffered 150 mM NaCl solution (2 ml/duct) at 37°C. Immotile or contaminated samples were discarded. Pooled spermatozoa were centrifuged at 600 × g for 4 min and washed twice with the same saline solution. Sperm concentration was determined during the second wash. 50 microliter aliquots of the sperm suspension were diluted with 1 ml of 0·4% Triton X-100 and counted in a Neubauer chamber. Spermatozoa pellets were resuspended to 35 million cells per ml, except when indicated, with MCM-PL medium (see below).

Induction of synchronous acrosome reaction

NaCl-washed spermatozoa were incubated at 37°C with minimal culture medium supplemented with pyruvate and lactate (MCM-PL) pH 7·8 (Mújica and Valdes-Ruiz, 1983). After 20 min, the calcium-ionophore A23187 was added at 1·3 μM final concentration. After 5–10 min, more than 90% of sperm cells showed acrosome loss.

Recovery of membrane vesicles released after the acrosome reaction

Once the acrosome reaction had occurred, several protease inhibitors were added to the MCM-PL medium: 4 mM PMSF, 4 mM pHMB, 4 mM pABA, 1 mM TLCK, 2 mM NEM and 5 μM leupeptin. Spermatozoa were separated by centrifugation at 3000 × g for 30 min at 4°C. The supernatant was saved and centrifuged at 100,000 × g for 60 min at 4°C. The pellet was resuspended with 1 mM EDTA, 50 mM Tris, pH 7·5, containing protease inhibitors and 1% (w/v) SDS final concentration.

Purification of acrosome-reacted sperm heads

A23187-induced acrosome-reacted sperm, resuspended with 1 mM EDTA, pH 5·0 and supplemented with a cocktail of protease inhibitors were sonicated for 3 min at 80 mA, using a microtip equipped Model B-12 Branson sonicator, as Oko and Clermont (1988) described. Flagellum and head split up was assessed by light microscopy. After sonication, the samples were centrifuged at 3000 × g at 4°C for 30 min. The pellet, containing separated heads and flagella, was washed three times with the EDTA-protease inhibitors solution. To purify the sperm heads, the washed pellet was resuspended with 1 ml of 65% sucrose 0·02 M PBS, pH 7·2. The 0·02 M PBS was prepared by diluting 1 ml of a PBS stock solution (140 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, 8·1 mM Na_2HPO_4) with 6·5 ml of distilled water. The re-suspended sperm heads were laid on top of a sucrose discontinuous density gradient and centrifuged at 100,000 × g for 70 min at 4°C. Gradients were prepared with 65, 70 and 75% (w/v) sucrose-PBS, 4 ml each. Heads were recovered from the bottom of the tube, resuspended to 1 ml with 50 mM Tris, pH 7·5, supplemented with protease inhibitors and washed three times by centrifugation at 3000 × g for 15 min at 4°C. Sample purity was assessed by phase contrast and electron microscopy.

Perinuclear material solubilization

Purified acrosome-reacted sperm heads, 50 million heads/ml of 50 mM Tris pH 9·0 plus protease inhibitors, were incubated in the presence of 0·2 ml 90 mM DTT for 15 min, then with 0·2 ml 10% CTAB in distilled water as described (Hernández-Montes *et al.*, 1973). Solubilized proteins and free nuclei were separated by centrifugation at 5000 × g for 30 min at 4°C. The nuclear pellet was washed with PBS and fixed in 2·5% glutaraldehyde. The supernatant proteins were precipitated with 5 volumes of cold acetone for 3 hr at −20°C and recovered by centrifugation at 5000 × g for 30 min at 4°C. The pellet was resolubilized with 4% SDS, 50 mM Tris, pH 7·5 and was resolved by SDS-10% polyacrylamide gel electrophoresis.

Membrane solubilization from intact non-capacitated spermatozoa

NaCl-washed spermatozoa were resuspended to 50 million/ml with a 1 mM EDTA solution, pH 5·0, containing protease inhibitors and sonicated for 30 sec at 4°C, as previously described (Aitken *et al.*, 1988).

Demembreanated sperm cells were pelleted by centrifugation at 3000 × g for 30 min at 4°C. The membrane fraction was recovered from the supernatant by centrifugation at 100,000 × g for 60 min 4°C. The pellet was resuspended in 250 *µl* of 1 mM EDTA, 50 mM Tris, pH 7·5 supplemented with protease inhibitors and 1% (w/v) SDS.

Electrophoresis and western blot of spermatozoa proteins

Aliquots from all the sperm fractions obtained were taken for protein determination (Lowry *et al.*, 1951) and for 10% polyacrylamide gel electrophoresis as Laemmli described (1970). Electrophoresis was run at 125 mv for 4 hr. Electrotransfer of proteins to nitrocellulose paper (0·25 *µm*) was performed at 90 mv during 3 hr (Towbin *et al.*, 1979). Calmodulin was transferred as Hincke (1988) reported.

Identification of calmodulin binding proteins

To block the non-specific calmodulin binding sites, sperm membrane protein blot was incubated at 37°C with 25 mM Tris, 150 mM NaCl pH 7·4 (TBS) supplemented with 0·05% Tween 20 (TBS-T) and 5% (w/v) fat-free milk. After 1 hr, it was washed with 50 ml of TBS-T. Calmodulin purified from bovine testis (250 *µg*) was dissolved either in 30 ml of TBS-T plus 2 mM $CaCl_2$ or in TBS-T plus 4 mM EDTA. Each sample of sperm membranes was blotted by duplicate, one was overlayed with Ca^{2+}-calmodulin and the other with EDTA-calmodulin, and incubated for 30 min at 37°C with gentle agitation. Non-bound calmodulin was discarded and the nitrocellulose paper was washed for 10 min, with 50 ml of TBS-T, three times. Goat or sheep anti-calmodulin antibodies, diluted in TBS-T plus 3% (w/v) BSA to 1 : 200 or 1 : 50 respectively, were added to the blots either in the presence of 2 mM $CaCl_2$ or 4 mM EDTA, and incubated at 37°C 2 hr with stirring. The excess of antibody was eliminated and the nitrocellulose paper was washed three times with 50 ml of TBS-T for 10 min. Gold-labeled protein A, dissolved 1 : 10 in TBS-T plus 3% BSA was employed as a second antibody. After 1 hr of incubation at 37°C the excess of protein A was eliminated and the nitrocellulose paper was washed as before. Since protein-A shows low affinity for sheep-IgG and goat-IgG (Johnston and Thorpe, 1982), it was necessary to use the immunogold/silver staining system (Danscher, 1981; Moeremans *et al.*, 1984; Brada and Roth, 1984).

The identification of the calmodulin binding proteins was also assayed in the following conditions: (a) using rabbit IgG anti-sheep (or anti-goat) IgG peroxidase conjugate as a second antibody and developed with DAB (De Blas and Cherwinski, 1983; Hauri and Bucher, 1986), (b) employing biotinylated calmodulin and the avidin-peroxidase developing system. Three negative controls were included: (a) incubation without calmodulin, (b) incubation with trifluoroperazine-treated calmodulin, and (c) using a preimmune serum as a first antibody. Rat liver mitochondria calmodulin binding proteins, identified under our experimental conditions, were included as a fourth control. To do this, mitochondria were isolated as described (Pardue *et al.*, 1981), resuspended in the presence of 5 mM PMSF, 2 mM TLCK, 5 mM NEM, 5 mM pABA and 20 *µ*M leupeptin, incubated with 25 mM DTT, 10 min at room temperature and sonicated in the presence of 2·2% (w/v) SDS. Aliquots of the whole solubilized material were resolved by electrophoresis and blotted to determine their calmodulin binding proteins.

Immunocytochemical localization of calmodulin

Aliquots of isolated sperm head suspension were fixed in 3% formaldehyde-PBS (v/v) for 60 min at room temperature and pelleted by centrifugation at 600 × g for 4 min. Pellets were washed three times with PBS, then incubated with 50 mM NH_4Cl for 10 min and rinsed again with PBS. After a distilled water wash, they were re-suspended in distilled water and smeared on slides. After being air-dried, the smears were incubated for 60 min at 37°C with sheep polyclonal anti-calmodulin antibody diluted 1 : 50 in PBS-1% BSA. After three rinses with PBS, the second antibody, rabbit anti-sheep IgG-FITC conjugate was added to the smears and were incubated at 37°C for 60 min. This was followed by two rinses with PBS. The smears were mounted using glycerol-PBS (v/v) pH 8·0 and examined under a Zeiss photomicroscope equipped for phase-contrast and fluorescence. Photographs were taken on Kodak Film Tri X Pan.

Sample preparation for electron microscopy

Aliquots of the washed cell fraction pellets were fixed for 90 min with 2·5% glutaraldehyde in 0·1 M cacodylate buffer pH 7·3, postfixed 90 min in 0·1% osmium tetroxide and processed for observation in a Jeol JEM 2000 EX electron microscope.

Results

Acrosome-reacted sperm head purification

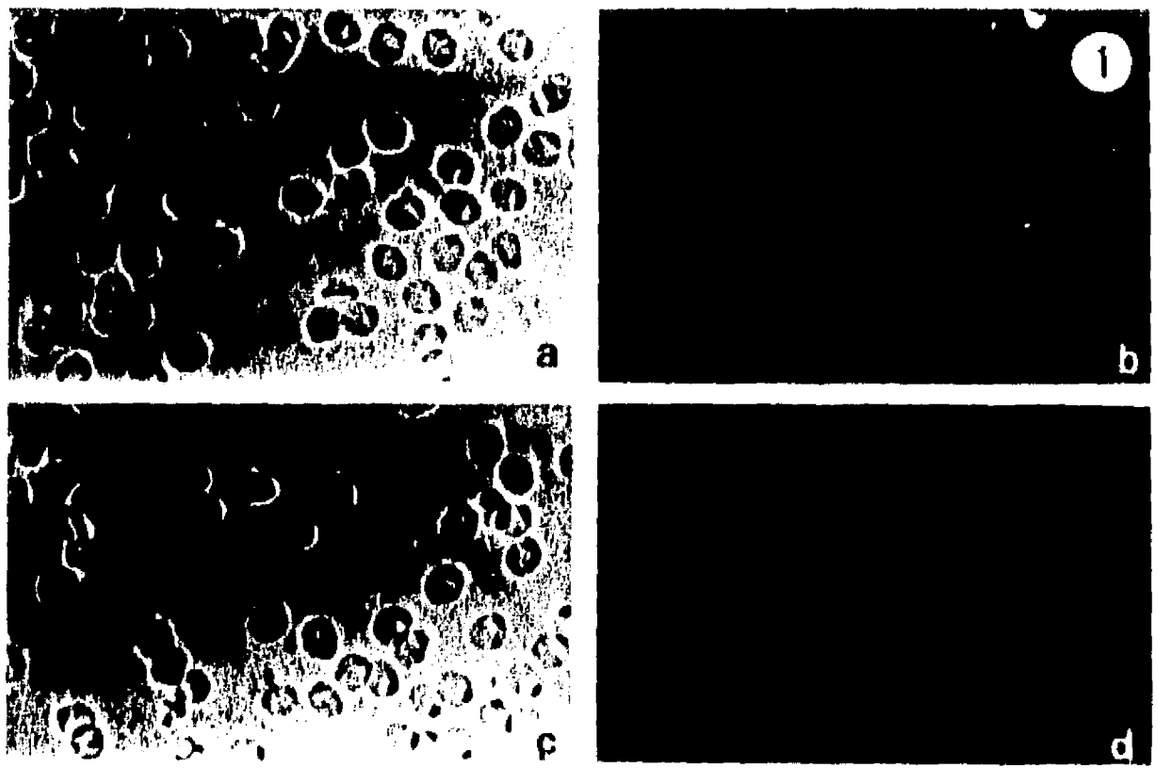
The sperm heads from A23187-induced acrosome-reacted spermatozoa were isolated with a high degree of purity: no residual fragments of flagella were observed under optical microscope examination of the samples obtained from three different experiments. A representative phase contrast image is shown in Figure 1a. When examined for calmodulin immunofluorescence, the isolated sperm heads showed a positive staining in the postacrosomal region (Fig. 1b). This result indicates that in spite of the sonication treatment and gradient separation, this region of the acrosome-reacted sperm preserved bound calmodulin (Trejo and Mújica, 1990). When the first antibody was adsorbed with calmodulin, a negative reaction was observed (Fig. 1d). Ultrastructural observation of these samples showed that the plasma membrane and acrosome were absent from spermatozoa after the sonication (Fig. 2a) and it was corroborated that the isolated head preparation was free of flagella fragments (Fig. 2b). Purified heads obtained after the sucrose density gradient centrifugation preserved an electron dense material in the postacrosomal region and a fuzzy substance in the mid-anterior nuclear region or subacrosomal layer (Fig. 2b). This envelope is lost after treatment with DTT-CTAB, producing a less condensed nude nuclei (Fig. 2c).

Calmodulin-binding proteins detection by immunoblot

To assess the identification of the calmodulin binding proteins in the different fractions obtained from intact and acrosome reacted spermatozoa, several internal controls were included. One of them was to prove that calmodulin was able to bind to the nitrocellulose paper and that it could be detected either from a pure preparation or from a mixture of proteins. Figure 3 shows the immunoblot of the bovine calmodulin used to overlay the sperm fractions (lane C) and of the guinea pig endogenous calmodulin from solubilized spermatozoa (lane D), both of them were detected using the sheep anti-calmodulin antibody and developed by the rabbit anti-sheep IgG labeled with peroxidase. Three negative controls were also included: overlay with trifluoroperazine-treated calmodulin, overlay without adding calmodulin and overlay with preadsorbed antibody. No labeled bands were observed when trifluoroperazine-treated calmodulin was used to overlay the sperm fractions (data not shown), in accordance with previous reports showing that this drug binds to calmodulin in a Ca^{2+}-dependent manner and that it blocks any further interaction of calmodulin with other targets (Klee, 1988). A lack of cross-reactivity between the sperm membrane proteins and the antibodies was observed, no labeled bands appeared after overlay without adding calmodulin when preadsorbed antibody was employed (data not shown). The identification of the calmodulin binding proteins in isolated rat liver mitochondria using unmodified calmodulin to overlay the blotted proteins, the sheep anti-calmodulin polyclonal antibody, the gold labeled protein A and the silver staining as developing system, was included as a positive control (Fig. 4). Six major Ca^{2+}-dependent calmodulin binding proteins, M_r = 63, 56, 51, 41, 38 and 35 kD were detected, (lane C), which resemble the molecular weight of the affinity purified mitochondria calmodulin binding proteins (Gazzotti *et al.*, 1984; Hatase *et al.*, 1983; Pardue *et al.*, 1981). Besides these bands, several minor proteins able to bind calmodulin in the presence of calcium were also detected (lane C). No labeled bands were seen in the absence of calcium (lane D).

Calmodulin binding proteins in solubilized membrane fractions from non capacitated sperm

The multiple proteins that could be resolved by PAGE of the membranes obtained from intact spermatozoa by sonication and solubilization with SDS are shown in Figure 5C. This fraction, constituted by the plasma membrane and the outer and inner acrosomal membranes, showed several polypeptides



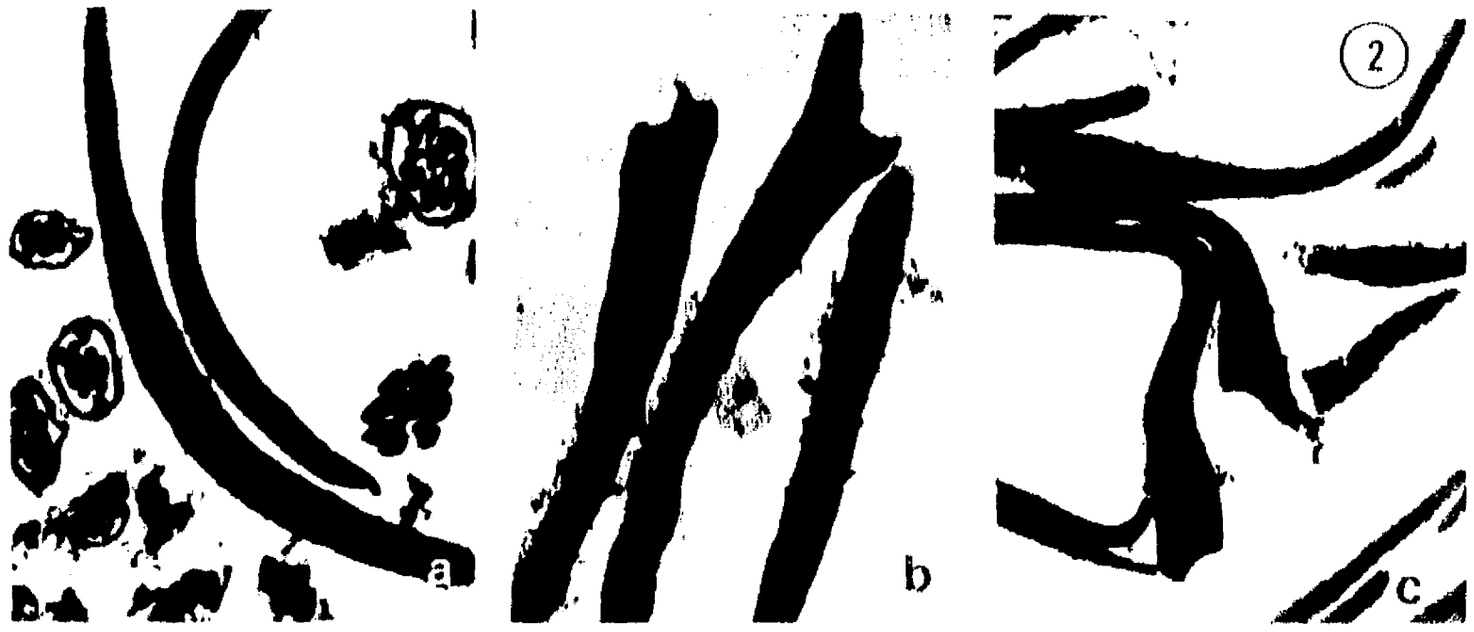


Fig. 1. Calmodulin localization in isolated sperm heads by indirect immunofluorescence. A23187-induced acrosome reacted spermatozoa were sonicated to split up heads and flagella and isolated heads were recovered after centrifugation on sucrose density gradients. Acrosome-reacted isolated heads were fixed with 3% formaldehyde-PBS. Calmodulin was localized by indirect immunofluorescence using a sheep polyclonal anticalmodulin antibody and an anti-sheep IgG labeled with fluorescein. (a) and (c) Phase contrast images. (b) Calmodulin immunolocalization in isolated sperm heads showing a pale fluorescence in the postacrosomal region and a stronger stain in the implantation fossa. (d) Negative control. The representative image observed when purified heads were incubated with preadsorbed anticalmodulin antibody and when a preimmune IgG substituted the anticalmodulin antibody. ×630.

Fig. 2. Electron microscopy images of acrosome-reacted isolated sperm heads. Once the acrosome reaction had occurred by action of the calcium ionophore A23187, a cocktail of protease inhibitor was added to the spermatozoa suspension before sonication, an aliquot of the sonicated spermatozoa was fixed with 2·5% glutaraldehyde. The heads and flagella were separated by centrifugation. Heads were purified using a discontinuous density sucrose gradient and centrifugation at 100,000 × g for 70 min at 4°C and then fixed. (a) Acrosome-reacted spermatozoa after sonication showing the absence of the plasma membrane and of the acrosome. (b) Isolated heads after centrifugation on sucrose gradient showing no contamination with flagella fragments or with other structural elements. Note that they preserved their perinuclear material. (c) Nuclei obtained after DTT-CTAB treatment of acrosome-reacted isolated heads. Observe that the perinuclear fuzzy envelope was lost after this treatment and nude nuclei were obtained. Fig. 2a ×18,000, Fig. 2b ×20,000; Fig. 2c ×20,000.

able to bind biotinylated and unmodified calmodulin probes in the presence (Fig. 6, lanes B and E) and in the absence of calcium (lanes C and F). Biotinylated calmodulin, revealed by the avidin-peroxidase method, was bound to several peptides both in the presence (lane B) and in the absence of Ca^{2+} (lane C). Although the calmodulin-binding proteins detected in both conditions showed a similar molecular weight, in the presence of Ca^{2+}, the proteins with M_r = 110 and 66 kD showed a stronger label compared with the 47, 45, 32 and 21–14 kDa bands (lane B). This might be due to differences in the level of each binding protein. Even though overlay with biotinylated calmodulin had been employed to detect the Ca^{2+}-dependent calmodulin-binding proteins in other tissues (Billingsley *et al.*, 1985; Pennypacker *et al.*, 1989), to rule out the possibility that this chemical modification of calmodulin would not allow the detection of the Ca^{2+}-dependent calmodulin binding proteins, unmodified calmodulin was overlaid to sperm protein blots either in the presence or in the absence of calcium. The calmodulin-binding proteins were revealed using the sheep anti-calmodulin and anti-IgG-horseradish peroxidase system. In the presence of calcium, two major proteins showed the ability to bind calmodulin, M_r = 47 and 45 kDa and several minor pale bands, 110, 66, 56 and a 32 kDa (lane D). In the absence of calcium, besides these bands, several low molecular weight peptides were also detected (lane E). Again, no Ca^{2+}-dependent calmodulin-binding proteins were detected in the membrane fraction obtained from intact spermatozoa. The results shown were observed in three experiments.

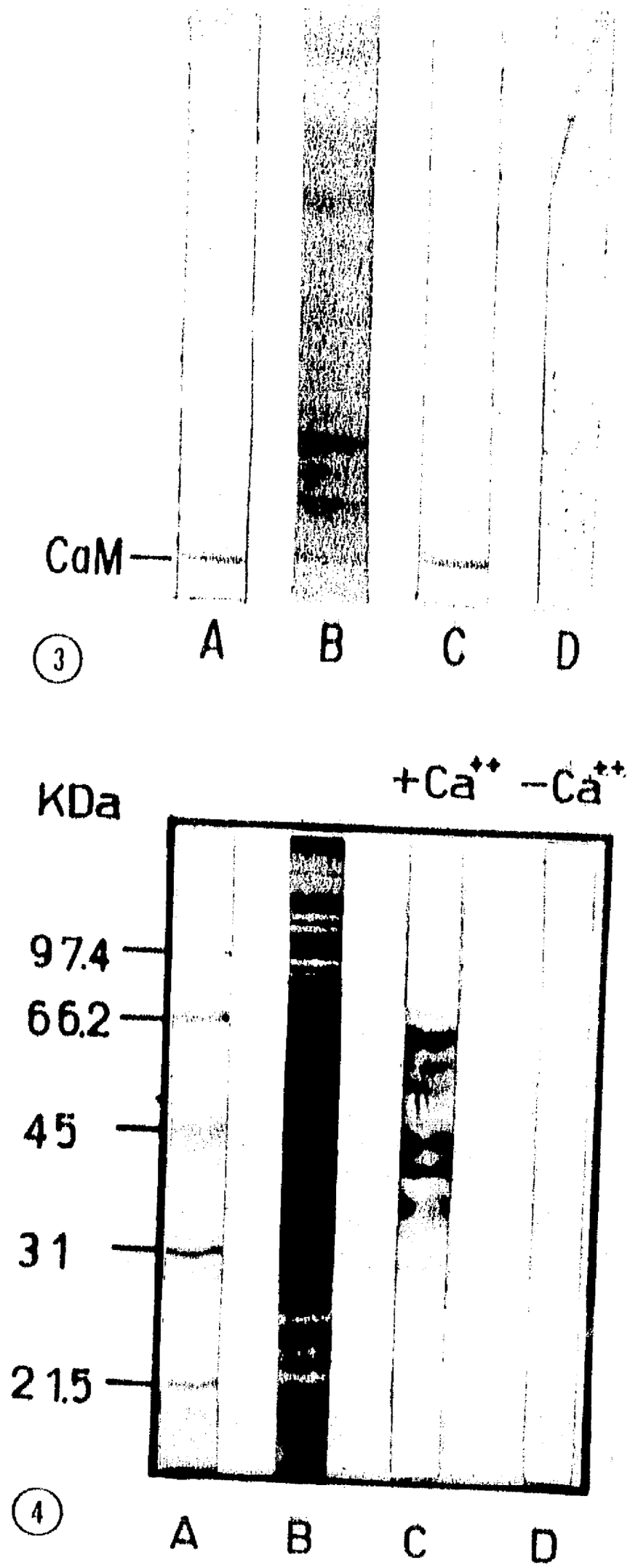
Although only Ca^{2+}-independent calmodulin binding proteins were shown using the two calmodulin probes, not all the same proteins were detected by the two methods and when it was the case, the label strength for each band was not equivalent. This variation may be the result of different affinity for unmodified and biotinylated calmodulin shown by the sperm membrane proteins, since non-specific labeling was ruled out by two internal controls: when biotinylated calmodulin was omitted and only avidin-horse radish peroxidase was used to overlay the blots, no labeled bands were detected, and also when a preimmune IgG, either from sheep or goat, substituted the anti-calmodulin antibody, no labeled bands appeared (one experiment, data not shown).

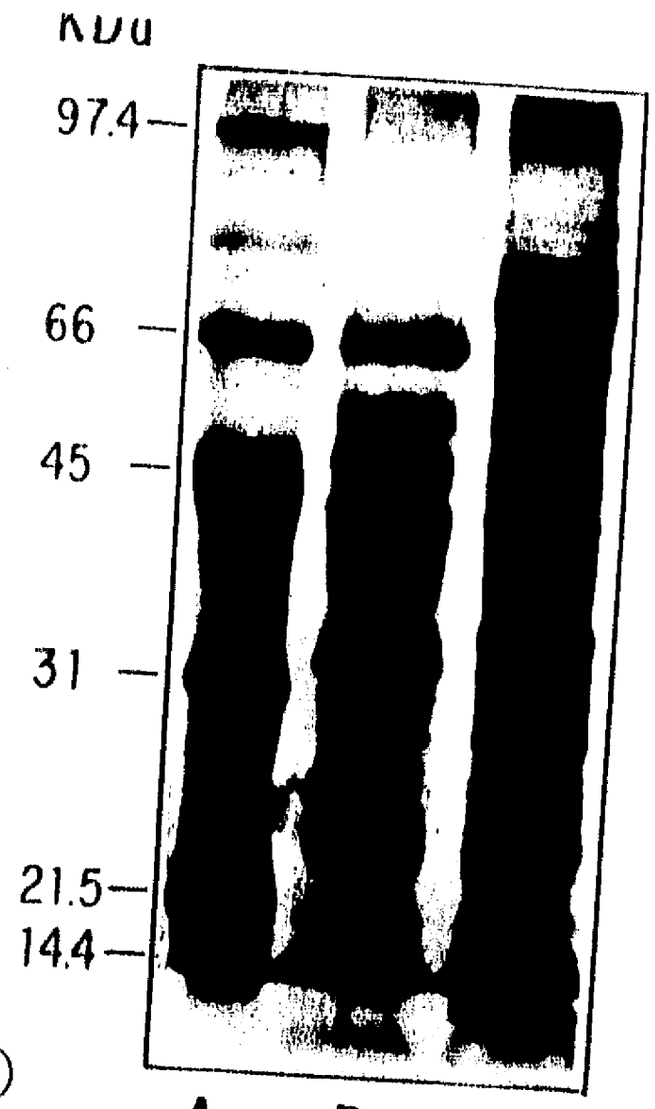
Calmodulin binding proteins in membrane vesicles released after the acrosome reaction

The membrane vesicles produced by the fusion of the plasma membrane and the outer acrosomal membrane during the A23187-induced acrosome reaction were observed under electron microscope and no con-

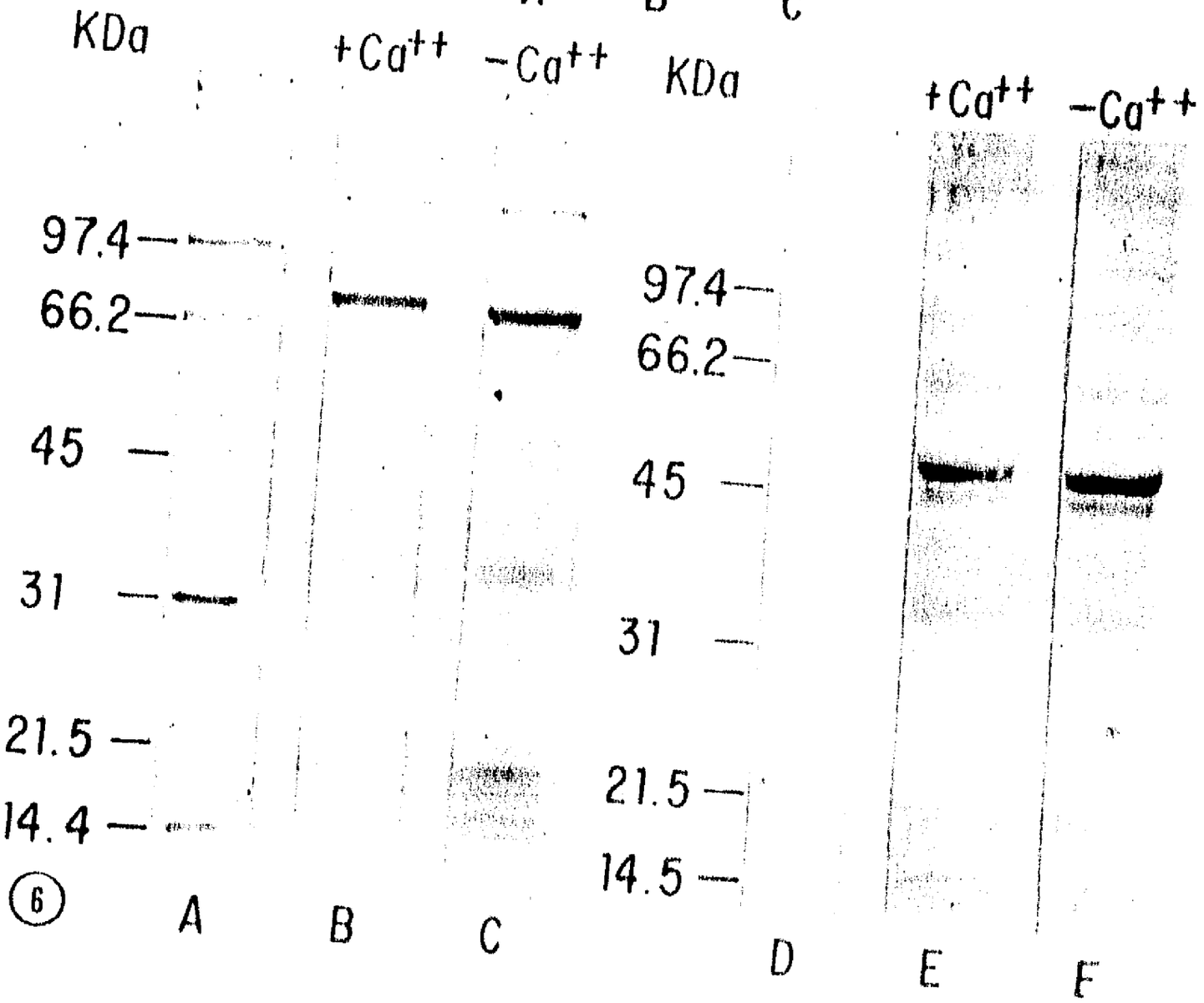
Fig. 3. Immunoblot of calmodulin. Bovine calmodulin purified from testis and solubilized non-capacitated spermatozoa samples were resolved by SDS–12% polyacrylamide gel electrophoresis (Laemmli, 1970), transferred to nitrocellulose paper as Hincke (1988) reported and stained with 0·1% (w/v) amido black in 10% (v/v) acetic acid. Incubation with sheep anticalmodulin polyclonal antibody and rabbit anti-sheep IgG labeled with peroxidase, allowed the detection of calmodulin in both samples. The typical results observed in three experiments are shown. (lane A) Purified bovine calmodulin. (lane B) Spermatozoa proteins stained with amido black. (lane C) Immunoblot of purified bovine calmodulin. (lane D) Immunodetection of endogenous guinea pig sperm calmodulin.

Fig. 4. Identification of calmodulin-binding proteins in rat liver mitochondria by overlay with unmodified calmodulin. Isolated rat liver mitochondria were sonicated and solubilized as spermatozoa samples. Solubilized proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose paper. Unmodified native bovine calmodulin was overlayed to the blotted proteins, either in the presence or in the absence of Ca^{2+} followed by incubation with sheep anticalmodulin antibody and with gold labeled-protein A. The calmodulin binding proteins were revealed by the gold/silver stain system. (Lane A) Molecular weight standards. (lane B) Mitochondrial proteins stained with amido black. (Lane C) Calmodulin-binding proteins detected by calmodulin overlay in the presence of Ca^{2+} and (lane D) in the absence of Ca^{2+}. Observe that several Ca^{2+}-dependent calmodulin binding proteins were detected and no labeled bands appeared when calmodulin overlay was made in the absence of Ca^{2+}. These results were observed in two determinations from the same mitochondrial sample.


CaM
③
A
B
C
D
KDa
+Ca++
−Ca++
97.4
66.2
45
31
21.5
④
A
B
C
D


KDa
97.4
66
45
31
21.5
14.4
A
B
C

⑤


KDa
+Ca++
−Ca++
97.4
66.2
45
31
21.5
14.4
A
B
C
KDa
97.4
66.2
45
31
21.5
14.5
D
+Ca++
−Ca++
E
F

⑥

tamination with other sperm structures was detected (Fig. 7). The membrane vesicle proteins resolved by SDS-electrophoresis and stained with amido black are shown in Fig 5 lane B.

Differences in calmodulin-binding proteins detection using biotinylated or unmodified calmodulin were observed in this fraction, also characterized by the few peptides able to bind calmodulin in a Ca^{2+}-independent and in a Ca^{2+}-inhibited manner. It was observed, in three different experiments, that two major peptides M_r = 110 and 66 kDa bound biotinylated calmodulin in the presence of Ca^{2+} (Fig 8 lane B) and four peptides M_r = 110, 97, 66, and 45 kDa without Ca^{2+} (Fig. 8 lane C). The presence of Ca^{2+}-independent calmodulin-binding proteins, M_r = 66 and 45 kDa, was confirmed by overlay with unmodified calmodulin in the presence (Fig. 8 lane E) and in the absence of Ca^{2+} (Fig. 8 lane F). However no Ca^{2+}-inhibited calmodulin binding proteins were detected with the unmodified probe. Thus, biotinylated calmodulin allowed the detection of two peptides M_r = 110 and 97 kDa able to

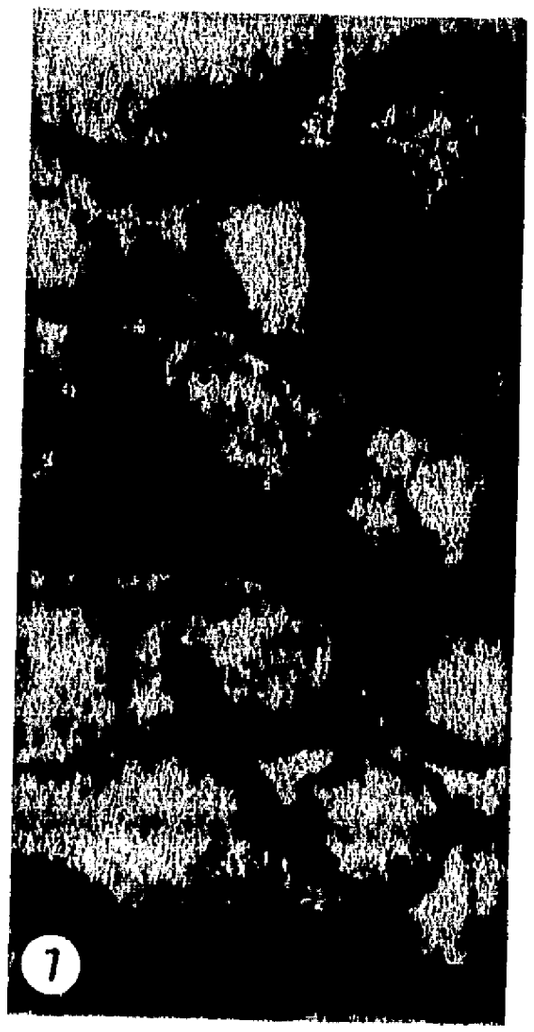
Fig. 5. Silver stain protein pattern of the sperm membrane fractions. Membrane vesicles produced by the fusion of the sperm plasma membrane and the outer acrosomal membrane and released during the A23187 induced acrosome reaction, were recovered by centrifugation at 100,000 × g for 60 min at 4°C. The membrane fraction obtained by sonication of non-capacitated spermatozoa, a mixture of plasma membrane and outer and inner acrosomal membranes, was also recovered by centrifugation at 100,000 × g. Aliquots containing 80 μg of protein from these two membrane fractions were resolved by SDS-10% polyacrylamide gel electrophoresis (Laemmli, 1970) and stained by the silver method of Morrissey (1981). Representative patterns from at least three experiments are shown. (A) Molecular weight standards, (B) vesicles released after the acrosome reaction and (C) membrane fraction obtained by sonication of non-capacitated spermatozoa.

Fig. 6. Identification of the calmodulin-binding proteins in the membrane fraction obtained from non-capacitated spermatozoa. NaCl-washed spermatozoa were sonicated in the presence of a cocktail of protease inhibitors. The membrane fraction was pelleted by centrifugation at 100,000 × g for 60 min at 4°C and solubilized with SDS. Proteins were separated by SDS-10%-polyacrylamide gel electrophoresis and blotted to nitrocellulose paper. Calmodulin binding proteins were detected by overlay either with biotinylated calmodulin (lanes B and C) in the presence and in the absence of Ca^{2+}, respectively, or with unmodified native calmodulin (lanes E and F) with and without Ca^{2+}, respectively. (Lanes A and D), molecular weight standards.

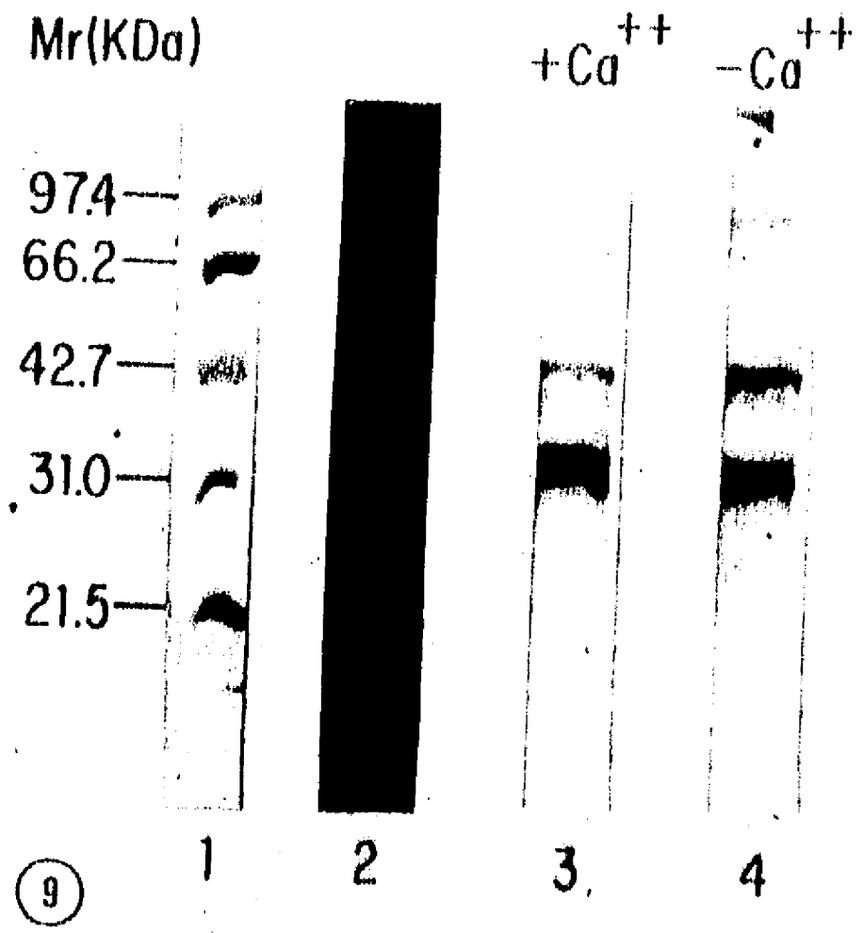
Fig. 7. Electron microscopy image of isolated acrosome reaction vesicles. The membrane vesicles released during the A23187-induced acrosome reaction were recovered and fixed with glutaraldehyde to examine it under the electron microscope. Vesicles of different sizes were observed and no other sperm structures were apparent. ×96,000.

Fig. 8. Identification of calmodulin-binding proteins in the vesicles released after the acrosome reaction. The proteins from the membrane vesicles produced by the fusion of the plasma membrane and the outer acrosomal membrane were tested for their ability to bind either biotinylated or unmodified bovine calmodulin, both in the presence and in the absence of calcium. The calmodulin binding proteins shown were observed in two different experiments. (Lanes A and D) Molecular weight standards. Lanes B and C show the polypeptides able to bind biotinylated calmodulin in the presence (lane B) and in the absence of calcium (lane C). Lanes E and F show the polypeptides to which unmodified calmodulin was bound in the presence (lane E) and in the absence of calcium (lane F).

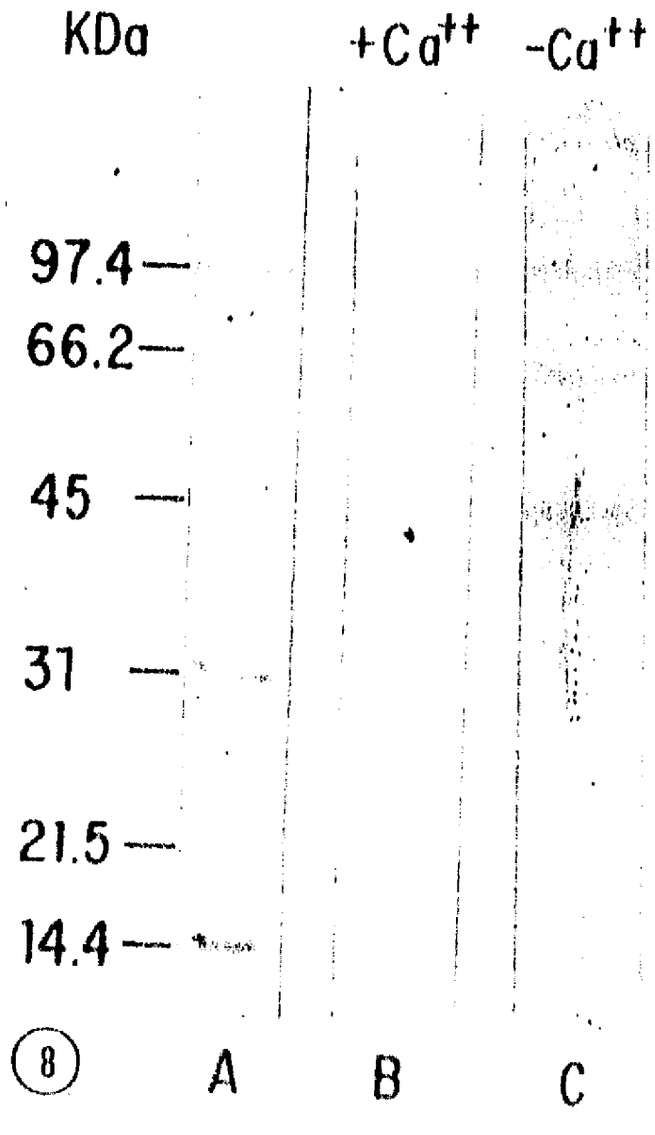
Fig. 9. Identification of the calmodulin-binding proteins in the perinuclear material of acrosome-reacted isolated heads. Purified sperm heads from A23187-induced acrosome-reacted spermatozoa were treated with DTT and CTAB to solubilize their fuzzy perinuclear envelope. An aliquot of the solubilized material was resolved by SDS-10% polyacrilamide gel electrophoresis, blotted to nitrocellulose paper and tested for their ability to bind unmodified calmodulin, either in the presence and in the absence of calcium. (Lanes 1) Molecular weight standards. (Lane 2) Amido black stain of the perinuclear solubilized proteins. Lane 3 shows the polypeptides able to bind calmodulin in the presence of Ca^{2+} and in lane 4, the proteins which bound calmodulin in the presence of EDTA, revealed by the double antibody system. These results were observed in three different experiments.



⑦


Mr(KDa)
+Ca++
-Ca++
97.4
66.2
42.7
31.0
21.5
1
2
3
4

⑨


KDa
+Ca++
-Ca++
97.4
66.2
45
31
21.5
14.4
A
B
C

⑧

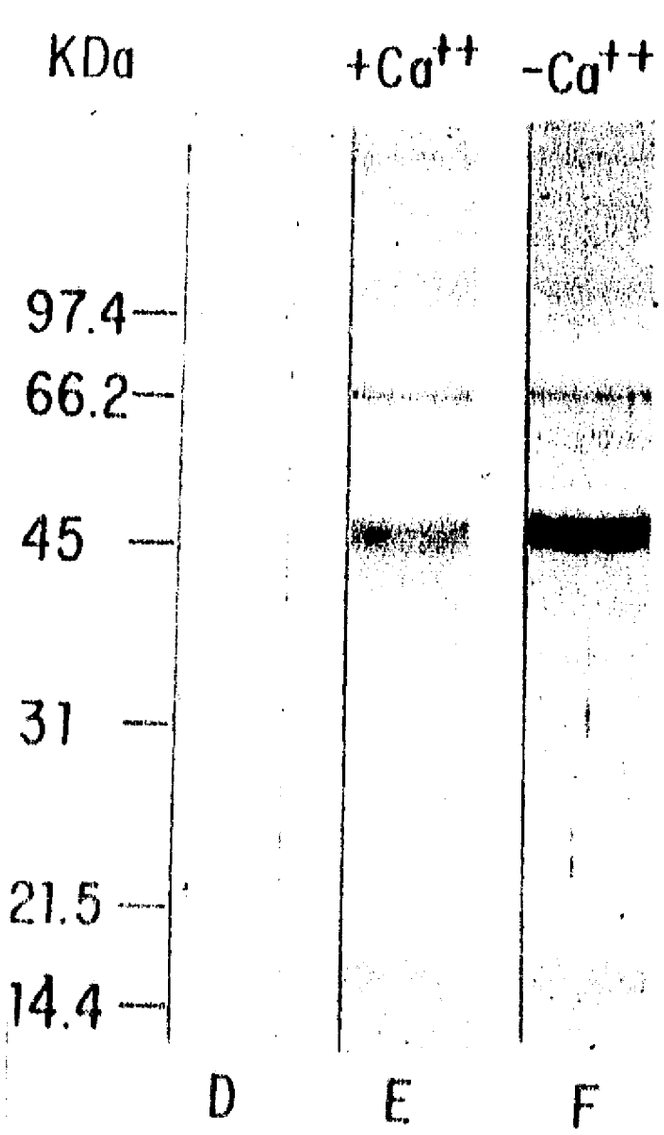

KDa
+Ca++
-Ca++
97.4
66.2
45
31
21.5
14.4
D
E
F

Table 1. *Calmodulin binding proteins in spermatozoa membrane.*

Species	Author	Ca^{2+}	Relative Molecular Weights (kDa)																	
Bull	(1) (2)	+	123													25	20		16	10
		−		93													20	18	16	10
Boar	(3)	+			67	65		52	46								21–17		14	
		−															21–47			
Human	(4)	+				64	59·5	54		44·8	34	32	30	27		22			11	
							59·1			44·8						24	18		12	
Guinea pig	(5)	+	110		66				47	45		32								
		−	110	97	66				47	45		32				22–14				

(1) Nolan *et al.* (1985) (2) Olson *et al.* (1985) (3) Peterson *et al.* (1989) (4) Aitken *et al.* (1988) (5) Present work
(+) In presence of calcium (−) Without calcium, with EGTA

interact with calmodulin in a Ca^{2+}-inhibited mode.

The calmodulin-binding proteins with similar molecular weight detected in both, solubilized plasma and acrosomal membranes from intact non acrosome-reacted spermatozoa and in the solubilized membrane vesicles released after the acrosome reaction, are probably the same. These are membrane proteins, a cytoplasmic origin seems unlikely, which preserved their capability to bind calmodulin in spite of the proteolytic enzyme release during the acrosome reaction. The calmodulin-binding proteins of low molecular weight detected in the membrane fraction from intact sperm, might belong to the inner acrosomal membrane, to the plasma membrane of other regions of the sperm not involved in the acrosome reaction or to have lost their ability to bind calmodulin by the action of the proteases released during this process.

Calmodulin binding proteins in the solubilized perinuclear material

The perinuclear material remaining in the sperm head after the acrosome reaction includes the nuclear membrane, the postacrosomal lamina and the perinuclear theca. The latter is a complex structure characterized by its resistance to extraction in nonionic detergents but solubilized by DTT-CTAB. The posterior portion of this structure shows a great morphological stability and a complex protein complement (Longo *et al.*, 1987). These characteristics were confirmed under the electron microscope examination of the isolated acrosome-reacted heads (Fig. 2b) and by the electrophoretic pattern shown by the solubilized perinuclear material (Fig. 9 lane 2). However, only two polypeptides showed the ability to bind unmodified calmodulin in the presence of Ca^{2+}, M_r = 43 and 34–31 kDa (lane 3) and three in its absence, with molecular weights 97, 43, 34–31 kDa (lane 4).

The immunolocalization of calmodulin in the postacrosomal region of isolated acrosome-reacted sperm heads (Fig. 1b), which lack plasma membrane, confirmed previous observations (Trejo and Mújica, 1990) and indicated that calmodulin was firmly associated with the postacrosomal matrix elements. It seems very likely that from this region proceed the 34, 43 and 97 kDa calmodulin binding peptides detected in the solubilized perinuclear material. Although the 34 and 43 kDa proteins might be similar to the proteins detected in the two membrane fractions analyzed, the 97 kDa band may also have an equivalent in those fractions, but was the only Ca^{2+}-inhibited calmodulin binding protein detected in this fraction. These results were observed in three different experiments.

Discussion

It had been observed that sperm contains three different types of calmodulin binding proteins: Ca^{2+}-dependent, Ca^{2+}-independent and Ca^{2+}-inhibited calmodulin-binding

proteins (Clash and Means, 1982, 1983; Moore and Dedman, 1981; Olson *et al.*, 1985; Noland *et al.*, 1985; Aitken *et al.*, 1988; Leclerc *et al.*, 1989, 1990; Manjunath *et al.*, 1993). In the membrane fractions studied in the present paper, only two classes of calmodulin-binding proteins were detected: the Ca^{2+}-independent calmodulin-binding proteins, which bound calmodulin both in the presence and in the absence of calcium, and the Ca^{2+}-inhibited calmodulin-binding proteins, which bound calmodulin only in the absence of calcium. Polypeptides of the third class, the Ca^{2+}-dependent calmodulin binding proteins were not detected. This result was carefully analyzed and the experimental conditions were re-examined to rule out the possibility of artifactual results.

Although the biotinylation of calmodulin has allowed both, the detection of calcineurin in the nanogram range and its activation, as well as, the identification of the calmodulin-binding proteins in other tissues by the overlay procedure (Billingsley *et al.*, 1985), it is known that some chemical modifications of calmodulin affect its ability to interact with one enzyme but not with other enzymes (Walsh and Stevens, 1977; Thiry *et al.*, 1985), therefore, unmodified calmodulin was also employed to avoid a possible non detection of other calmodulin-binding proteins. Since the use of unmodified calmodulin to overlay mitochondrial blotted proteins allowed the detection of Ca^{2+}-dependent modulator binding proteins similar to those previously identified by affinity chromatography (Gazzotti *et al.*, 1984; Pardue *et al.*, 1981; Hatase *et al.*, 1983), and no Ca^{2+}-independent or Ca^{2+}-inhibited calmodulin-binding proteins were detected, the overlay with this calmodulin probe seemed to rule out a possible failure in the identification of the proteins able to bind calmodulin in the sperm membrane fractions studied. However, the detection of calmodulin-binding proteins by overlay procedures depends on the renaturation of a previously denaturated protein by detergent (SDS) (Glenney and Weber, 1980), it is possible that the calmodulin-binding proteins detected in the sperm membrane fractions represent only those polypeptides which recovered, at least partially, their native state and are present at a level within the sensitivity range of this method.

On the other hand, the different proteins detected by the two calmodulin probes and the label strength observed with the two methods deserve further discussion. It has been reported that five of the seven lysine residues are localized in the NH_2-portion of calmodulin, some of them have been involved in the connection of the two halves of the molecule (Newton *et al.*, 1984) whether the number and position of biotin molecules covalently bound to calmodulin modify its conformation change in the presence of calcium and therefore its interaction with other calmodulin-binding proteins besides calcineurin has not been defined. Previous studies have shown that calmodulin contains multiple interactive sites for different enzymes (Kuznicki *et al.*, 1981; Newton *et al.*, 1984; Klevit and Vanaman, 1984; Putkey *et al.*, 1986). Based on this, calmodulin binding proteins have been classified in three groups (Ni and Klee, 1985). One class of calmodulin-binding proteins, like phosphodiesterase and cAMP-dependent protein kinase, interacts with both the COOH- and NH_2-terminal portions of calmodulin. A second group of proteins interacts with the COOH-terminal portion but fails to recognize the NH_2-terminal half, calcineurin is a member of this class of proteins. A third class of calmodulin-binding proteins, like the calmodulin-stimulated protein kinase, interact only with the COOH-terminal half but this interaction is reversed at high ionic strength even in the presence of Ca^{2+} (Ni and Klee, 1985). Whether the calmodulin-binding proteins identified in the sperm membrane fractions studied using biotinylated calmodulin belong, as calcineurin, to the class of proteins able to interact with the COOH-half of calmodulin remains to be clarified.

The ability to bind calmodulin in the absence of calcium that have been observed by different authors in whole, intact solubilized spermatozoa or in subcellular fractions of mammalian spermatozoa (Table I), is not restricted to this cell type. The 110 kDa protein of the intestinal brush-border (Gleney and Weber, 1980), liver phosphorylase kinase (Cohen *et al.*, 1978), muscle troponin I (Olwin *et al.*, 1982), brain P-57 (Andreasen *et al.*, 1983; Cimler *et al.*, 1985) and bacterial adenylate cyclase (Greenlee *et al.*, 1982; Kilhoffer *et al.*, 1983) can interact with calmodulin in the absence of Ca^{2+} and it has been postulated that these proteins

may represent an additional class of proteins that interacts with calmodulin in a similar manner. Therefore, the 110 and the 97 kDa proteins detected in the acrosome reaction vesicles and the 97 kDa polypeptide identified in the perinuclear material of acrosome reacted isolated heads may be considered as a members of this group.

The brain Ca^{2+}-independent calmodulin binding protein, P-57, which shows higher affinity for calmodulin in the absence of Ca^{2+} ($K_d = 2\ \mu M$) than in its presence, has been suggested as a targeting protein to localize calmodulin at specific sites within the cell or on the inner side of the membrane through interaction with cytoskeletal components (Cimler *et al.*, 1985). It has also been postulated that at low free calcium concentration, a significant fraction of calmodulin will be bound to P-57 (Cimler *et al.*, 1985). Whether the Ca^{2+}-independent calmodulin-binding proteins identified in the membrane fractions from guinea pig sperm have a similar role should be clarified.

Although several factors have been involved in the control of sperm domains (Myles *et al.*, 1984; Gaunt *et al.*, 1983; Cowan *et al.*, 1987), sperm cytoskeleton may be involved in their maintenance. Actin has been localized in different regions of the sperm (Clark and Yanagimachi, 1978; Lora-Lamia *et al.*, 1986; Flaherty *et al.*, 1986; Flaherty *et al.*, 1988; Fouquet *et al.*, 1989), but the appearance of calmodulin and F-actin in the postacrosomal sperm region, after the acrosome reaction had taken place (Trejo and Mújica, 1990; Moreno-Fierros *et al.*, 1992), suggests that calmodulin may be involved in the structural organization of this domain, known to be involved in the sperm-egg membrane fusion. The adequate localization of proteins and receptors involved in egg recognition, postulated to be present in the postacrosomal region (Primakoff *et al.*, 1987), might be dependent on cytoskeletal elements. Calmodulin has been involved in the control of cytoskeleton through the participation of proteins able to bind it (Kakiuchi and Sobue, 1983; Kakiuchi, 1985), whether the calmodulin binding proteins detected in the solubilized perinuclear material are elements of the cytoskeletal-membrane complex was not clarified in this work.

This is the first report identifying the calmodulin binding proteins present in the membrane vesicles released and in the perinuclear material from acrosome-reacted sperm. Whether the Ca^{2+}-independent calmodulin binding proteins detected in these fractions are responsible for calmodulin compartmentation, or participate in membrane fusion and in membrane receptor localization in the postacrosomal region remain to be defined.

Acknowledgements

This research was partly supported by Consejo Nacional de Ciencia y Tecnología, grant P228CCOX891518. H.E.O. was supported by a doctoral fellowship (No. 54318), by Consejo Nacional de Ciencia y Tecnología. We are grateful to the staff of the Unidad de Microscopía Electrónica, CINVESTAV, for providing fluorescence microscope facilities.

References

Aitken, R. J., Ross, A., Hargreave, T., Richardson, D. and Best, F. 1984. Analysis of human sperm function following exposure to the ionophore A23187. *J. Androl.*, **5**, 321–329.

Aitken, R. J., Clarkson, J. S., Hulme, M. J. and Henderson, C. J. 1988. Analysis of calmodulin acceptor proteins and the influence of calmodulin antagonists on human spermatozoa. *Gamete Res.*, **21**, 93–111.

Andreasen, T. J., Luetje, C. W., Heideman, W. and Storm, D. R. 1983. Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry*, **22**, 4615–4618.

Bar-Sagi, D. and Prives, J. 1983. Trifluoroperazine, a calmodulin antagonist, inhibits muscle cell fusion. *J. Cell Biol.*, **97**, 1375–1380.

Billingsley, M. L., Pennypacker, K. R., Hooveer, C. G., Brigati, D. J. and Kincaid, R. L. 1985. A rapid and sensitive method for detection and quantification of calcineurin and calmodulin-binding proteins using biotinylated calmodulin. *Proc. Natl. Acad. Sci. USA.*, **82**, 7585–7589.

Brada, D. and Roth, J. 1984. 'Golden blot-detection' of polyclonal and monoclonal antibodies bound to antigens on nitrocellulose by protein A-gold complexes. *Anal. Biochem.*, **142**, 79–83.

Cimler, B. M., Andreasen, T. J., Andreasen, K. J. and Storm, D. R. 1985. P-57 is a neural specific calmodulin binding protein. *J. Biol. Chem.*, **260,** 10784–10788.

Clarke, G. N. and Yanagimachi, R. 1978. Actin in mammalian sperm heads. *J. Exp. Zool.*, **205,** 125–132.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. and Nairn, A. C. 1978. Identification of the Ca^{2+}-dependent modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase. *FEBS Letters*, **92,** 287–293.

Cowan, A. E., Myles, D. G. and Koppel, D. E. 1987. Lateral diffusion of the PH-20 protein on guinea pig sperm: Evidence that barriers to diffusion maintain plasma membrane domains in mammalian sperm. *J. Cell Biol.*, **104,** 917–923.

Danscher, G. 1981. Localization of gold in biological tissue. *Histochem.*, **71,** 81–88.

De Blas, A. L. and Cherwinski, H. M. 1983. Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies. *Anal. Biochem.*, **133,** 214–219.

Dedman, J. R., Welsh, M. J. and Means, A. R. 1978. Ca^{2+}-dependent regulator: production and characterization of a monospecific antibody. *J. Biol. Chem.*, **253,** 7515–7521.

Dedman, J. R. and Kaetzel, M. A. 1983. Calmodulin purification and fluorescent labelling. In *Methods in Enzymology* (eds. A. R. Means and B. W. O'Malley), Vol. 102, pp. 1–12. Academic Press, New York.

DeLorenzo, R. J. 1980. Role of calmodulin in neurotransmitter release and synaptic function. *Ann. N. Y. Acad. Sci.*, **356,** 92–109.

Flaherty, S. P., Winfrey, V. P. and Olson, G. E. 1986. Localization of actin in mammalian spermatozoa: a comparison of eight species. *Anat. Rec.*, **216,** 504–515.

Flaherty, S. P., Winfrey, V. P. and Olson, G. E. 1986. Localization of actin in human, bull, rabbit, and hamster sperm by immunoelectron microscopy. *Anat. Rec.*, **221,** 599–610.

Fouquet, J. P., Kann, M. L. and Dadoune, J. P. 1989. Immunogold distribution of actin during spermiogenesis in the rat, hamster, monkey, and human. *Anat. Rec.*, **223,** 35–42.

Fraser, L. R. 1977. Motility patterns in mouse spermatozoa before and after capacitation. *J. Exp. Zool.*, **202,** 439–444.

Fraser, L. R. 1987a. Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization *in vitro*. *J. Reprod. Fert.*, **81,** 77–89.

Fraser, L. R. 1987b. Extracellular calcium and fertilization related events. In *The Role of Calcium in Biological Systems* (ed. L. J. Anghileri), Vol. IV, pp. 163–190. CRC Press, Boca Raton.

Gaunt, S. J., Brown, C. R. and Jones, R. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. *Exp. Cell Res.*, **144,** 275–284.

Gazzotti, P., Gloor, M. and Carafoli, E. 1984. Calmodulin binding proteins in rat liver mitochondria. *Biochem. Biophys. Res. Commun.*, **119,** 343–351.

Glenney, J. R. Jr. and Weber, K. 1980. Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. *J. Biol. Chem.*, **255,** 10551–10554.

Gopalakrishna, R. and Anderson, W. B. 1982. Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.*, **104,** 830–836.

Greenlee, D. V., Andreasen, T. J. and Storm, D. R. 1982. Calcium-independent stimulation of *Bordetella pertussis* adenylate cyclase by calmodulin. *Biochemistry*, **21,** 2759–2764.

Hatase, O., Tokuda, M., Sharma, R. K., Wang, J. H. and Green, D. E. 1983. Purification and characterization of the heat-stable calmodulin-binding protein from the matrix of bovine heart mitochondria. *Biochem. Biophys. Res. Commun.*, **113,** 633–637.

Hauri, H. P. and Bucher, K. 1986. Immunoblotting with monoclonal antibodies: Importance of blocking solution. *Anal. Biochem.*, **159,** 386–389.

Hernández-Montes, H., Iglesias, G. and Mújica, A. 1973. Selective solubilization of mammalian spermatozoa structures. *Exp. Cell. Res.*, **76,** 437–440.

Hidaka, H., Yamaki, T., Totsuka, T. and Asano, M. 1979. Selective inhibitors of Ca^{2+}-binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin interaction. *Mol. Pharmacol.*, **15,** 49–59.

Hidaka, H., Yamaki, T., Naka, M., Tanaka, T., Hayashi, H. and Kobayashi, R. 1980. Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmacol.*, **17,** 66–72.

Hincke, M. T. 1988. Conditions for improved adsorption of calmodulin to nitrocellulose: Detection by ^{45}Ca binding. *Electrophoresis*, **9,** 303–306.

Johnstone, A. and Thorpe, R. 1982. Affinity chromatography and immunoprecipitation. In *Immunochemistry in Practice* (eds. A. Johnstone and R. Thorpe.), pp. 202–232. Blackwell Scientific Publications, Oxford.

Jones, H. P., Bradford, M. M., McRorie, R. A. and Cormier, M. J. 1978. High levels of a calcium-dependent modulator protein in spermatozoa and its similarity to brain modulator protein. *Biochem. Biophys. Res. Commun.*, **82,** 1264–1272.

Kakiuchi, S. and Sobue, K. 1983. Control of the cytoskeleton by calmodulin and calmodulin-binding proteins. *Trends Biochem. Sci.*, **8**, 59–62.

Kakiuchi, S. 1985. Biochemistry of the Ca^{2+} and calmodulin-dependent regulation of the cytoskeleton. In *Calcium and Cell Physiology* (ed. D. Marmé), pp. 227–237. Springer-Verlag, Berlin; New York.

Kilhoffer, M. C., Hope-Crokk, G. and Wolff, J. 1983. Calcium-independent activation of adenylate cyclase by calmodulin. *Eur. J. Biochem.*, **133**, 11–15.

Klee, C. B. 1988. Interaction of calmodulin with Ca^{2+} and target proteins. In *Calmodulin. Molecular Aspects of Cellular Regulation* (eds. P. Cohen, C. B. Klee), Vol. 5, pp. 35–56. Elsevier Science Publishers B. V., Amsterdam.

Klevit, R. E. and Vanaman, T. C. 1984. Azidotyrosylcalmodulin derivatives. Specific probes for protein-binding domains. *J. Biol. Chem.*, **259**, 15414–15424.

Kopeikina-Tsiboukidou, L., and Deliconstantinos, G. 1986. Calcium-induced membrane metabolic alterations modify the sex steroids binding into dog brain synaptosomal plasma membranes. *Int. J. Biochem.*, **18**, 777–784.

Kopeikina-Tsiboukidou, L. and Deliconstantinos, G. 1989. Calmodulin selectively modulates the guanylate cyclase activity by repressing the lipid phase separation temperature in the inner half of the bilayer of rat brain synaptosomal plasma membranes. *Neurochem. Res.*, **14**, 119–127.

Kuznicki, J., Grabarek, Z., Brzeska, H. and Drabikowski, W. 1981. Stimulation of enzyme activities by fragments of calmodulin. *FEBS Letters*, **130**, 141–145.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Leclerc, P., Langlais, J., Lambert, R. D., Sirard, M. A. and Chafouleas, J. G. 1989. Effect of heparin on the expression of calmodulin-binding proteins in bull spermatozoa. *J. Reprod. Fert.*, **85**, 615–622.

Leclerc, P., Sirard, M. A., Chafouleas, J. G. and Lambert, R. D. 1990. Decreased binding of calmodulin to bull sperm proteins during heparin-induced capacitation. *Biol. Reprod.*, **42**, 483–489.

Lenz, R. W. and Cormier, M. J. 1982. Effects of calmodulin-binding drugs on the guinea pig spermatozoon acrosome reaction and the use of these drugs as vaginal contraceptive agents in rabbits. *Ann. N. Y. Acad. Sci.*, **383**, 85–97.

Levin, R. M. and Weiss, B. 1979. Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *J. Pharmacol. Exp. Ther.*, **208**, 454–459.

Longo, F. J., Krohne, G., Franke, W. W. 1987. Basic proteins of the perinuclear theca of mammalian spermatozoa and spermatids: a novel class of cytoskeletal elements. *J. Cell. Biol.* **105**, 1105–1120.

Lora-Lamia, C., Castellani-Ceresa, L., Andreetta, F., Cotelli, F. and Brivio, M. 1986. Localization and distribution of actin in mammalian sperm heads. *J. Ultrastruct. Mol. Struct. Res.*, **96**, 12–21.

Lowry, O. H., Rosebrough, N. J., Farr, L. and Randall, R. L. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Lydan, M. A. and O'Day, D. H. 1988. Different developmental functions for calmodulin in Dictyostelium: trifluoperazine and R24571 both inhibit cell and pronuclear fusion but enhance gamete formation. *Exp. Cell Res.*, **178**, 51–63.

Manjunath, P., Chandonnet, L., Baillargeon, L. and Roberts, K. D. 1993. Calmodulin-binding proteins in bovine semen. *J. Reprod. Fert.*, **97**, 75–81.

Moeremans, M., Daneels, G., Van Dijck, A., Langanger, G. and De Mey, J. 1984. Sensitive visualization of antigen-antibody reactions in dot and blot immune overlay assays with immunogold and immunogold/silver staining. *J. Immunol. Meth.*, **74**, 353–360.

Moore, P. B. and Dedman, J. R. 1984. Calmodulin, a calmodulin acceptor protein, and calcineurins: unique antibody localizations in hamster sperm. *J. Cell Biochem.*, **25**, 99–107.

Moreno-Fierros, L., Hernández, E. O., Salgado, Z. O. and Mújica A. 1992. F-actin in guinea pig spermatozoa: its role in calmodulin translocation during acrosome reaction. *Mol. Reprod. Devel.*, **33**, 172–181.

Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.*, **117**, 307–310.

Mújica, A. and Valdes-Rufz, M. A. 1983. On the role of glucose in capacitation and acrosomal reaction of guinea pig sperm. *Gamete Res.*, **8**, 335–344.

Myles, D. G., Primakoff, P. and Koppel, D. E. 1984. A localized surface protein on guinea pig sperm exhibits free diffusion on its domain. *J. Cell Biol.*, **98**, 1905–1909.

Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J. and Klee, C. B. 1984. Agonist and antagonist properties of calmodulin fragments. *J. Biol. Chem.*, **259**, 4419–4426.

Ni, W-C. and Klee, C. B. 1985. Selective affinity chromatography with calmodulin fragments coupled to sepharose. *J. Biol. Chem.*, **260**, 6974–6981.

Noland, T. D., Van Eldik, L. J., Garbers, D. L. and Burgess, W. H. 1985. Distribution of calmodulin and calmodulin-binding proteins in membranes from bovine epididymal spermatozoa. *Gamete Res.*, **11**, 297–303.

Oko, R. and Clermont, Y. 1988. Isolation, structure and protein composition of the perforatorium of rat spermatozoa. *Biol. Reprod.*, **39**, 673–687.

Olson, G. E., Winfrey, V. P., Garbers, D. L. and Noland, T. D. 1985. Isolation and characterization of a macro-

molecular complex associated with the outer acrosomal membrane of bovine spermatozoa. *Biol. Reprod.* **33**, 761–779.

Olwin, B. B., Keller, C. H. and Storm, D. R. 1982. Interaction of a fluorescent N-danzylaziridine derivative of troponin I with calmodulin in the absence and presence of calcium. *Biochemistry*, **21**, 5669–5675.

Ortego, F., Riquelme, G. and Lackington, I. 1985. Interaction of calmodulin antagonists with plasma membrane and with plasma membrane lipid. In *Calmodulin Antagonists and Cellular Physiology* (eds. H. Hidaka and D. J. Hartshorne), pp. 321–333. Academic Press, New York.

Pardue, R. L., Kaetzel, M. A., Hahn, S. H., Brinkley, B. R. and Dedman, J. R. 1981. The identification of calmodulin-binding sites on mitochondria in cultured 3T3 cells. *Cell*, **23**, 533–542.

Pennypacker, K. R., Kincaid, R. L., Polli, J. W. and Billingsley, M. L. 1989. Expression of calmodulin-dependent phosphodiesterase, calmodulin-dependent protein phosphatase, and other calmodulin-binding proteins in human SMS-KCNR neuroblastoma cells. *J. Neurochem.*, **52**, 1438–1448.

Peterson, R. N., Chaudry, P. and Tibbs, B. 1989. Calcium-binding proteins of boar spermatozoan plasma membranes: identification and partial characterization. *Gamete Res.*, **23**, 49–60.

Primakoff, P., Hyatt, H. and Tredick-Kline, J. 1987. Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion. *J. Cell Biol.*, **104**, 141–149.

Putkey, J. A., Dractta, G. F., Slaughter, G. R., Klee, C. B., Cohen, P., Stull, J. T. and Means, A. R. 1986. Genetically engineered calmodulins differentially activate target enzymes. *J. Biol. Chem.*, **261**, 9896–9903.

Sano, K. 1983. Inhibition of the acrosome reation of sea urchin spermatozoa by a calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide (W-7). *J. Exp. Zool.*, **226**, 471–473.

Tanaka, T., Inagaki, M., Ito, M. and Hidaka, H. 1985. Calmodulin antagonists and protein kinase C. In *Calmodulin Antagonists and Cellular Physiology* (eds. H. Hidaka and D. J. Hartshorne), pp. 525–529. Academic Press, New York.

Tash, J. S. and Means, A. R. 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. *Biol. Reprod.*, **26**, 745–763.

Tash, J. S. and Means, A. R. 1983. Cyclic adenosine 3',5'monophosphate, calcium and protein phosphorylation in flagellar motility. *Biol. Reprod.*, **28**, 75–104.

Thiry, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J. and Christophe, J. 1980. The activation of brain adenylate cyclase and brain cyclic-nucleotide phosphodiesterase by seven calmodulin derivatives. *Eur. J. Biochem.*, **103**, 409–414.

Trejo, R. and Mújica, A. 1990. Changes in calmodulin compartmentalization throughout capacitation and acrosome reaction in guinea pig spermatozoa. *Mol. Reprod. Devel.*, **26**, 366–377.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.*, **76**, 4350–4354.

Umekawa, H., Gaka, M., Inagaki, M. and Hidaka, H. 1985. Interaction of W-7, a calmodulin antagonist with another Ca^{2+}-binding protein. In *Calmodulin Antagonists and Cellular Physiology* (eds. H. Hidaka and D. J. Harsthorne), pp. 511–524. Academic Press, New York.

Walsh, M. and Stevens, F. C. 1977. Chemical modification studies on the Ca^{2+}-dependent protein modulator of cyclic nucleotide phosphodiesterase. *Biochemistry*, **16**, 2742–2749.

Weinman, S., Ores-Carton, C., Raintean, D. and Puszkin, S. 1986. Immunoelectron microscopic localization of calmodulin and phospholipase A_2 in spermatozoa. I. *J. Histochem. Cytochem.*, **34**, 1171–1179.

Yanagimachi, R. 1982. Requirements of extracellular calcium ions for various stages of fertilization and fertilization-related phenomena in the hamster. *Gamete Res.*, **5**, 323–344.

Yanagimachi, R. 1988. Mammalian fertilization. In *Physiology of Reproduction* (eds. E. Knobil, J. D. Neil, L. L. Ewing, G. S. Greenwald, C. L. Markert and D. W. Pfaff), Vol. 1. pp. 135–185. Raven Press, New York.

Yanagimachi, R. and Usui, N. 1974. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Exp. Cell Res.*, **89**, 161–174.

CONCLUSIONES Y PERSPECTIVAS

A diferencia de lo observado en células inducidas a proliferar, los cambios en el contenido de CaM testicular no fueron transitorios y no se asociaron a una fase pre-replicativa de DNA. Los incrementos en el contenido de CaM del testículo en desarrollo se asociaron a la aparición de nuevas generaciones de espermatocitos primarios, a la realización de la meiosis y a la aparición de las espermátides tempranas. Esta situación plantea la necesidad de identificar las señales que controlan la expresión de los genes que codifican a CaM durante la meiosis.

Por otra parte, el paralelismo entre el aumento en el contenido de CaM y sus proteínas blanco observado durante la fase de crecimiento exponencial del testículo, sugiere la existencia de mecanismos que coordinan al sistema CaM-proteínas aceptoras, que dependen de la velocidad de proliferación, previamente propuesta por otros autores en células transformadas.

La migración de CaM en espermatocitos primarios tardíos y en espermátidas tempranas sugiere que CaM podría estar mediando funciones semejantes en el núcleo de estos dos tipos celulares.

Con base en observaciones que demuestran que la expresión de algunos genes es modificada a través de la fosforilación de proteínas que se asocian al DNA y de factores de transcripción, realizada por la proteína cinasa II dependiente de Ca^{2+}-CaM y la presencia de proteínas aceptoras de CaM en las partículas de RNA heterogéneo nuuclear (hnRNA), se ha propuesto que CaM participa en la regulación de la expresión genética a dos niveles, en la síntesis de RNAm y en su procesamiento.

Estos dos procesos tienen singular importancia durante la espermatogénesis. Por una parte, durante la meiosis, específicamente en los espermatocitos primarios que han alcanzado el estadio paquiteno, se expresan genes específicos de testículo. El RNAm puede ser traducido en la proteína correspondiente en el propio espermatocito o bien ser almacenado y procesado después de la meiosis, hasta que se alcanzan estadios definidos de la espermiogénesis. Las espermátidas también pueden expresar genes específicos del testículo, pero sólo en estadios tempranos de su maduración. La síntesis de proteínas es muy activa en los espermatocitos primarios y en las espermátidas en fase Gogi, capuchón y acrosomal. Sin embargo, la síntesis de RNAm y de proteínas disminuye hasta desaparecer en las espermátidas tardías.

Una de las preguntas más importantes que quedan por definir es la participación de CaM en el control de la expresión genética de las células espermatogénicas. La localización nuclear de CaM y la presencia de proteín-cinasas dependientes de Ca^{2+}-CaM en los espermatocitos primarios tardíos, refuerza la posibilidad de que las células germinales masculinas pudieran presentar mecanismos de regulación genética dependientes de Ca^{2+}-CaM.

El papel de CaM en las funciones nucleares puede extenderse a la reparación del DNA. Se ha demostrado que la administración de drogas con actividad anticalmodulina potencian los efectos de agentes quimioterapeúticos que alquilan al DNA porque bloquean la reparación del DNA. Las proteínas dependientes de CaM que participan en este proceso no han sido identificadas. Los espermatocitos primarios presentan grandes ventajas para el estudio de la reparación del DNA. Alrededor del 0.1 al 0.2 % del DNA total es replicado mediante este mecanismo durante el estadio de paquiteno (Stern, 1986). El estudio y la manipulación experimental de este proceso en los espermatocitos primarios, podría dar información aplicable a otros tipos celulares, incluyendo células cancerosas, cuya reparación del DNA se ha alterado.

La demostración de que se requieren niveles adecuados de CaM para una segregación equitativa de los cromosomas en las células hijas, plantea la interrogante del mecanismo a través del cual CaM pudiera controlar este proceso. La separación de las cromátides hermanas y la transición metafase II/anafase II es inducida por Ca^{2+}-CaM en los ovocitos de rana a través de la estimulación de la actividad del sistema proteolítico responsable de la degradación de la ciclina (Lorca y cols., 1991, 1993, 1994). Sin embargo, el comportamiento cromosómico cambia durante la meiosis. En la primera división meiótica se separan los cromosómas homólogos y en la segunda división meiótica se separan cromátidas hermanas, como sucede en la mitosis. Si CaM participa en la separación del par de cromosomas homólogos a través del mismo mecanismo que dispara la segregación de cromátides hermanas, no se ha determinado.

Además de las funciones nucleares, el papel de CaM en el control del citoesqueleto de las células germinales queda abierto a investigaciones futuras. Las proteínas aceptoras de CaM purificadas del citosol de los espermatocitos primarios en este trabajo, parecen ser parte del citoesqueleto de estas células. Sin embargo, se requiere profundizar en su caracterización para definir su identidad y determinar su función. No existen reportes en la

literatura donde se hayan estudiado las proteínas aceptoras de Ca^{2+}-CaM en estas células, con los cuales se pueda comparar la información obtenida en este trabajo. Por otra parte, la identificación de los elementos que constituyen el citoesqueleto de las células germinales es muy limitada. Aunque se han estudiado los tipos de actina y tubulina presentes en las células espermatogénicas, no se han caracterizado las proteínas asociadas a los microtúbulos ni la regulación de estos componentes con los otros constituyentes del citoesqueleto. Además de la constitución del flagelo, el citoesqueleto dependiente de los microtúbulos sufre cambios muy importantes durante la diferenciación de las espermátidas. La polimerización de los microtúbulos y la formación de los tubos caudales durante la fase acrosomal y su despolimerización durante las etapas de maduración de las espermátidas, requiere de estudio para definir qué señales se asocian para ensamblar y desensamblar estas estructuras y el papel de CaM en estos procesos.

La participación de elementos asociados al citoesqueleto en la migración de organelos durante la espermiogénesis y la formación del espermatozoide con dominios funcionales y estructurales específicos, requiere de un estudio muy profundo. En etapas tempranas de la espermiogénesnis, el retículo endoplásmico forma una doble membrana alrededor del flagelo en formación y alrededor de los tubos caudales, pero al igual que estas estructuras, desaparece casi por completo en estadios de maduración más avanzados. Las mitocondrias migran de la periferia celular hacia el flagelo y forman una sola estructura que rodea la porción proximal del flagelo del espermatozoide. Las vesículas membranales con restos citoplásmicos migran a lo largo del flagelo y son desprendidos como gota citoplásmica. El sistema de transporte intracelular encargado de estas funciones no ha sido estudiado. La importancia de este proceso en la producción de gametos viables subraya la necesidad de su caracterización.

El enfoque experimental desde el cual se abordó el estudio de la participación de CaM en la reacción acrosomal del espermatozoide de mamífero, no permitió identificar aceptores específicos de CaM dependientes de Ca^{2+}. Se requiere definir la causa de los cambios en la distribución de CaM durante la reacción acrosomal. La gran movilidad que muestra, abre la pregunta de si migra asociada a una misma proteína o si por los cambios en la concentración de Ca^{2+}, puede asociarse a distintas proteínas que la reconocen parcial o

totalmente saturada de Ca^{2+}. Cambios en la concentración libre de Ca^{2+} limitados a regiones específicas, pudieran alterar su asociación a distintas proteínas en los dominios del espermatozoide. Los estudios donde se demuestra que el uso de drogas antiCaM pueden inducir la reacción acrosomal, sugieren que el contenido de CaM del espermatozoide es muy alto, ya que no es neutralizado por la concentraciones de droga empleadas. O bien que basta un nivel bajo de esta proteína en forma activa para disparar una cascada de eventos que se apoya en otras proteínas no activadas por CaM.

El objetivo principal de esta tesis **definir el papel de CaM en las células germinales masculinas a través de la caracterización de sus proteínas aceptoras** fue parcialmente alcanzado, hace falta definir la función de las proteínas aceptoras de CaM en las células germinales.

BIBLIOGRAFIA

Aitken, R.J., Clarkson, J.S., Hulme, M.J., Henderson, C.J. (1988). Analysis of calmodulin acceptor proteins and the influence of calmodulin antagonsits on human spermatozoa. Gamete Res. 21:93-111.

Andreasen, T.J., Luetje, C.W., Heideman, W., Storm, D.R. (1983). Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. Biochemistry 22:93-111.

Babu Y.S., Sack, J.S., Greenbough T.J, Bugg, C.E., Menas, A.R., Cook W.J. (1985). Three-dimensional structure of calmodulin. Nature 315:37-40.

Babu, Y.S., Bugg, C. E., Cook, W.J. (1988). Three-dimensional structure of calmodulin. En "Molecular Aspects of Cellular Regulation". Vol. 5. Calmodulin. P. Cohen & C.B. Klee (Eds). Elsevier Science Publishing Co. Netherlands. pp83-89.

Baitinger, C., Alderton, J., Poenie, M., Schulman, H., Steinhardt, R.A. (1990). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase is necessary for nuclear envelope breakdown. J. Cell Biol. 111:1763-1773.

Camatini, M., Anelli, G., Casale, A. (1986). Immunocytochemical localization of calmodulin in intact and acrosome-reacted boar sperm. Eur. J. Cell Biol. 41:89-96.

Campbell, A.K. (1983). Intracellular calcium. Its universal role as a regulator. John Wiley and Sons Ltd.

Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E. III, Means, A.R. (1982). Calmodulin and the cell cycle: involvement in regulation of cell-cycle progression. Cell 28:41-50.

Cheung W.Y. (1970). Cyclic 3',5'-nucleotide phosphodiesterase: demonstration of an activator. Biochem. Biophys. Res. Commun. 38:533-538.

Cheung W.Y. (1980). Calmodulin plays a pivotal role in cellular regulation. Science 207:19-27.

Cimier, R.M., Andreasen, T.J., Andreasen, K.I., Storm, D.R. (1985). P-57 is a neural specific calmodulin binding protein. J. Biol. Chem. 260:10784-10788.

Cohen, P (1988). The calmodulin-dependent multiprotein kinase. En "Molecular aspects of cellular regulation". Vol 5 Calmodulin. Eds. P. Cohen y C.B. Klee. Elsevier Science Publishing Co., Inc. Netherlands. Cap. 8. pp 145-196.

Colomer, J., López-Girona, A., Agell, N., Bachs, O. 81994).

Calmodulin regulates the expression of CDKS, cyclins and replicative enzymes during proliferative activation of human T lymphocytes. Biochem. Biophys. res. commun. 200:306-312.

Cox, J.A. (1984). Sequential events in calmodulin on binding with calcium and interaction with target enzymes. Federation Proc 43:3000-3004.

Crouch, T.H., Klee, C.B. (1980). Possitive cooperative binding of calcium to bovine brain calmodulin. Biochem 19:3692-3698.

Davis, T.N. (1986). A temperature-sensitive calmodulin mutant loses viability during mitosis. J. Cell Biol. 118:607-617.

Davis, T.N., Urdea, M.S., Masiarz, F.R., Thorner, J. (1986). Isolation of the yeast calmodulin gene: Calmodulin is an essential protein. Cell 47:423-431.

DeRemer, M.F., Saeli, R.J., Edelman, A.M. (1992). Ca^{2+}-calmodulin-dependent protien kinase Ia and Ib from rat brain. I. Identification, purification, and structural comparisons. J. Biol. Chem. 267:13460-13465.

DeRemer, M.F., Saeli, R.J., Brautigan, D.L., Edelman, A.M. (1992a). Ca^{2+}-calmodulin-dependent protien kinase Ia and Ib from rat brain. II. Enzymatic characteristics and regulation of activities by phosphorylation and dephosphorylation. J. Biol. Chem. 267:13466-13471.

Ebashi, S., Endo, M. (1968). Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. 18:123-183.

Gopalakrishna, R., Anderson, W.B. (1982). Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem. Biophys. res. Commun. 104:830-836.

Greaser, M.L., Gergely, J. 81971). Reconstitution of troponin activity from three protein components. J. Biol. Chem. 246:4226-4233.

Hodgkin, A.L., Keynes, R.D. (1957). Movements of labeled calcium in squid giant axons. J. Physiol. 138:253-281.

James P., Vorherr, T., Carafoli, E. (1995). Calmodulin-binding domains:just two faced or multi-faceted?. TIBS 20:38-42.

Jones, H.P., Bradford, M.M., McRorie, R.A., Cormier, M.J. (1978). High levels of a calcium-dependent modulator protein in spermatozoa and its similarity to brain modulator protein. Biochem. Biophys. Res. Commun. 82:1264-1272.

Jones, H.P., Lenz, R.W., Palevitz, B.A., Cormier, M.J. (1980). Calmodulin localization in mammalian spermatozoa. Proc. Natl. Acad. Sci. USA 77:2772-2776.

Kakiuchi, S., Yamasaki, R., Nakajima, H. (1970). Properties of a heat-stable phosphodiesterase activating factor isolated from brain extract. Studies on cyclic 3′,5′-nucleotide phosphodiesterae II. Proc. Jpn. Acad. 46:587-592.

Klee, C. B. (1977). Conformational transition accompanying the binding of Ca^{2+} to the protein acitvator of 3′,5′-cyclic adenosine monophosphate phosphodiesterase. Biochem 16:1017-1024.

Kilhoffer, M-C., Hope, G., Wolff, J. (1983). Calcium-independent activation of adenylate cyclase by calmodulin. Eur. J. Biochem. 133:11-15.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W. (1981). Stimulation of enzyme activities by fragments of calmodulin. FEBS Lett 130:141-145.

Lagacé, L., Chafouleas, G., Trejo, R., Delhumeau-Ongay, G., Dedman, J.R. (1981). Calmodulin levels during rat testis development and intracellular localization. Biol. Reprod. 22 (Suppl. 1):53A.

LaPorte, D.C., Weinman, B.M., Storm, D.R. (1980). Calcium-induced expossure of a hydrophobic surface on calmodulin. Biochem 19:3814-3819.

Lee, J.C., Edelman, A.M. (1995). Activation of Ca^{2+}-calmodulin-dependent protien kinase Ia is due to direct phosphorylation by its activator. Biochem. Biophys. res. Commun. 210:631-637.

Lenz, R.W., Cormier. M.J. (1982). Effects of calmodulin-binding drugs on the guinea pig spermatozoon acrosome reaction and the use of these drugs as vaginal contraceptive agents in rabbits. Ann New York Acad. Sci. 383:85-97.

López-Girona, A., Colomer, J., Pujol, M.J., Bachs, O., Agell, N. (1992). Calmodulin regulates DNA polymerase α activity during proliferative activation of NRK cells. Biochem. Biophys. Res. Commun. 184:1517-1523.

López-Girona, A., Bachs, O., Agell, N. (1995). Calmodulin is involved in the induction of DNA polymerases α and δ activities in normal rat kidney cells activated to proliferate. Biochem. Biophys. Res. Commun. 217:566-574.

Lorca, T., Galas, S., Fesquet, D., Devault, A., Cavadore, J-C., Dorée, M. (1991). Degradation of the proto-oncogene product $p39^{mos}$ is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca^{2+}-calmodulin dependent event. EMBO

J 10:2087-2093.

Lorca, T., Cruzalegui, F.H., Fesquet, D., Cavadore, J-C. Méry, J., Means, A.R., Dorée, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. Nature 366:270-273.

Lorca, T., Abrieu, A., Means, A., Dorée, M. (1994). Ca^{2+} is involved through type II calmodulin-dependent protein kinase in cyclin degradation and exit from metaphase. Biochim. Biophys. Acta 1223:325-332.

Means, A.R., Dedman, J.R. (1980). Calmodulin, an intracellular calcium receptor. Nature 285:73-77.

Meador, W.E., Means, A.R., Quiocho, F.A.(1992). Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. Science 257:1251-1255.

Meador, W.E., Means, A.R., Quiocho, F.A. (1993). Modulation of calmodulin plasticity in molecular recognition on the basis of X-ray structures. Science 262:1718-1721.

Miyano, O, Kameshita, I., Fujisawa, H. (1992). Purification and characterization of a brain-specific multifunctional calmodulin-dependent protein kinase from rat cerebellum. J. Biol. Chem. 267:1198-1203.

Mochizuki, H., Ito, T., Hidaka, H. (1993). Purification and characterization of Ca^{2+}/Calmodulin-dependent protein kinase V from rat cerebrum. J. Biol. Chem. 268:9143-9147.

Morin, N., Abrieu, A., Lorca, T., Martin, F., Dorée, M. 81994). The proteolysis-dependent metaphase to anaphase transistion: calcium/calmodulin-dependent protein kinase II mediates onset of anaphase in extracts prepared from unfertilized *Xenopus* eggs. EMBO J 13:4343-4352.

Moriya, M., Katagir, C., Yagi, K. (1993). Immuno-electron microscopic localization of calmodulin and calmodulin-binding proteins in the mouse germ cells during spermatogenesis and maturation. Cell Tissue Res. 271:441-451.

Nagae, T., Srivastava, P.N. (1986). Induction of the acrosome reaction in guinea pig spermatozoa by calmodulin antagonist W-/. Gamete Res. 14:197-208.

Nairn A.C., Bhagat, B., and Palfrey, H.C. (1985). Identification of calmodulin-dependent protein kinase III and its major M_r 100,000 substrate in mammalian tissues. Proc. natl. Acad. Sci. 82:7939-7943,

Nairn, A.C., Palfrey, H.C. (1987). Identification of the major M_r 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. J. Biol. Chem. 262:17299-17303.

Newton, D.L., Oldewurtel, M.D., Krinks, M.H., Shiloach, J., Klee, C.B. (1984). Agonist and antagonist properties of calmodulin fragments. J. Biol. Chem. 259:4419-4426.

Ni, W-C., Klee, C.B. (1985). Selective affinity chromatography with calmodulin fragments coupled to sepharose. J. Biol. Chem. 260:6974-6981.

Ohya,Y., Anraku, Y. (1989). A galactose-dependent *cmd1* mutant of *Saccharomyces cerevisiae*: involvement of calmodulin in nuclear division. Curr. Genet. 15:113-120.

O'Neil, K.T., DeGrado, W.F. (1990). How calmodulin binds its targets: sequence independent recognition of amphiphilic -helices. TIBS 15, 59-64.

Piñol, M.R., Berchtold, M.W., Bachs, O., Heizmann, C.W. (1988). Increased calmodulin synthesis in the pre-replicataive phase of rat liver regeneration. FEBS Lett. 231:445-450.

Pujol, M.J., Soriano, M., Aligué, R., Carafoli, E., Bachs, O. (1989). Effect of ∝-adrenergic blockers on calmodulin association with the nuclear matrix of rat liver cells during proliferative acitvation. J. Biol. Chem. 264: 18863-18865.

Richman P. G., Klee C.B (1979): Specific perturbation by Ca^{2+} of tyrosyl residue 138 of calmodulin. J. Biol. Chem. 254:5372-5376.

Selbert, M.A., Anderson, K.A., Huang, Q-H., Goldsterin, E.G., Means, A.R., Edelman, A.M. (1995). Phosphorylation and activation of Ca^{2+}-calmodulin-dependent protein kinase IV by Ca^{2+}-calmodulin-dependent protein kinase Ia kinase. J. Biol. Chem. 270:17616-17621.

Sano, K. (1983). Inhibition of the acrosome reaction of sea urchin spermatozoa by a calmdoulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). J. Exptl. Zool. 226:471-473.

Serratosa, J., Pujol, M.J., Bachs, O., Carafoli, E. (1988), Rearrangement of nuclear calmodulin during proliferative liver cell activation. Biochem. Biophys. res. Commun. 150:1162-1169.

Slaughter, G.R., Needlman, D.S., Means, A.R. (1987). Developmental regulation of calmodulin, actin, and tubulin RNAs during rat testis differentiation. Biol. Reprod. 37:1259-1270.

Slaughter, G.R., Meistrich, M.L., Means, A,R. (1989). Expression of RNAs for calmodulin, actins, and tubulins in rat testis cells.

Biol. Reprod. 40:395-405.

Slaughter,G.R., Means, A.R. (1989). Analysis of expression of multiple genes encoding calmodulin during spermatogenesis. Mol. Endocrinol. 3:1569-1578.

Smoake, J.A., Song, S.Y., Cheung, W.Y. (1974). Cyclic 3',5'-nucleotide phosphodiesterase distribution and developmental changes of the enzyme and its protein activator in mammalian tissues and cells. Biochim. Biophys. Acta 341:402-411.

Stern, H. (1986). Meiosis: some considerations. J. Cell Sci. Suppl. 4:29-43.

Takeda, T., Yamamoto, M. 81987). Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA 84:3580-3584.

Tanaka, T., Hidaka, H. (1980). Hydrophobic regions function in calmodulin-enzyme(s) interaction. J. Biol. Chem. 255:11078-11080.

Trejo, R., Turner, R., Dedman, J.R. (1985). Changes in calmodulin mRNA during rat spermatogenesis. J. Cell Biol.101:369a.

Rasmussen, C.D., Means, A.R. (1987). Calmodulin is involved in regulation of cell proliferation. EMBO J. 6:3961-3968.

Rasmussen, C.D., Means, A.R. (1989). Calmodulin is required for cell-cycle progression during G_1 and mitosis. EMBO J. 8:79-82.

VanEldik, L.J., Burgess, W.H. (1983). Analytical subcellualr distribution of calmodulin and calmodulin-binding proteins in normal and virus-transformed fibroblasts. J. Biol. Chem. 258:4539-4545.

Veigl, M.L., Vanaman, T.C., Sedwick, W.D. (1984). Calcium and calmodulin in cell growth and transformation. Biochim. Biophys. Acta 738:21-48.

Vogel, H.J., Huque, E.M., Hiraoki, T. (1990). Characterization of the surface of calmodulin by NMR. En " The biology and Medicine of Signal Transduction". Ed. Y Nishizuka y colaboradores. Raven Press, New York. pp254-259.

Walsh, M.P., Vallet, B., Autric, F., Demaille, J.G. (1979). Purificataion and characterization of bovine cardiac calmodulin-dependent myosin light chain kinase. J. Biol. Chem. 254;12136-12144.

Weinstein, H., Mehler, E.L. (1994). Ca^{2+}-binding and structural dynamics in the functions of calmodulin. Annu. Rev. Physiol. 56:213-236.

Werth, D.K., Hathaway, D.R., Watanabe, A. (1982). Regulation of phsophorylase kinase in rat ventricular myocardium. Role of calmodulin. Circ. Res. 51:448-456.

Wolff, D.J., Siegel, F.L. (1972). Purification of a calcium-binding phosphoprotein from pig brain. J. Biol. Chem. 247:4180-4185.