CALCIO INTRACELULAR: I. CARACTERIZACION DE LA UNION DEPENDIENTE
DE CALCIO DE LA GLUTAMATO DESCARBOXILASA CEREBRAL A LIPOSOMAS.
II. CUANTIFICACION DE LOS CAMBIOS EN LA CONCENTRACION DEL CALCIO
LIBRE INTRASINAPTOSOMAL USANDO ARSENAZO III.

TRABAJO DE TESIS QUE PRESENTA EL   M. EN C.

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INTRODUCCION

Desde los estudios de Ringer (Ringer, 1882) en relación con la importancia del Ca^{2+} extracelular en la excitabilidad del músculo cardíaco, se ha descubierto que este catión tiene una función central en la comunicación celular a diferntes niveles (Scarpa y Carafoli, 1978).

Algunas de las funciones biológicas del Ca son (Ashley y Campbell, 1979; Duncan, 1976):

1) Función estructural. El Ca se encuentra como precipitados de fosfato de Ca en estructuras esqueléticas (Montgomery et al., 1977); y el Ca también se encuentra unido a fosfolípidos y a proteínas para el ensamblaje y estabilización de la membrana biológica (Gitler, 1972).

2) Cofactor de enzimas extracelulares. La vía del complemento requiere de Ca durante la lisis celular inducida por anticuerpos (Porter y Reid, 1978). El Ca también es cofactor de la transformación de protrombina a trombina durante la coagulación de la sangre (Montgomery, et al., 1977).

3) Función eléctrica del Ca. El Ca funciona como estabilizador y como acarreador de corriente en membranas excitables (Frankenhauser y Hodgkin, 1957; Baker, 1972).

4) El Ca libre intracelular ($Ca^{2+}i$) como un regulador. En la tabla 1 se muestran algunas de las actividades celulares que dependen de la concentración de $Ca^{2+}i$, así como los estimulos

T A B L A   1

E S T I M U L O	R E S P U E S T A
NEUROTRANSMISOR	CONTRACCION MUSCULAR
DESPOLARIZACION DE LA TERMINAL NERVIOSA; Y LA ACCION DE NEUROTRANSMISORES Y HORMONAS EN GLANDULAS ENDOCRINAS Y EXO CRINAS	SECRECION
HORMONAS (GLUCAGON Y ADRENALINA)	GLUCOGENESIS, GLUCOGENOLISIS Y LIPOLISIS
LUZ	VISION
ESPERMA	FERTILIZACION
DESPOLARIZACION DE LA MEMBRANA	CAMBIOS EN LA PERMEABILIDAD A IONES

que las desencadenan. Se ha demostrado que diferentes estimulos inducen cambios en la concentración de $Ca^{2+}i$, por lo que se ha sugerido que este catión funciona como un 'mensajero' intracelular (Rasmussen, 1970).

El $Ca^{2+}i$, a diferencia de otros cationes intracelulares, tiene dos propiedades particulares: a) la concentración de $Ca^{2+}i$ es mantenida a niveles muy bajos (10 nM - 1 uM) en relación a la concentración extracelular (de 3 - 5 ordenes de magnitud de diferencia); y b) durante la actividad celular el $Ca^{2+}i$ fluctua sobre esos niveles muy bajos, y por lo tanto su concentración debe ser regulada muy eficientemente, ya que diferentes reacciones intracelulares son sensibles a cambios en la concentración de Ca^{2+} en el rango micromolar (Carafoli y Crompton, 1978).

Entre los cationes más abundantes y biologicamente importantes, como el Na^+, el K^+ y el Mg^{2+}, el Ca^{2+} tiene ciertas propiedades fisicoquímicas que le permitieron ser seleccionado dureante la evolución como un regulador y un 'mensajero intracelular:

Los iones monovalentes se mueven rapidamente y en gran proporción a través de la membrana, pero sus interacciones con otras moleculas en la fase acuosa y en las interfases son relativamente débiles. Por otro lado los cationes trivalentes (si bien no no se encuentran en los organismos vivos) interaccionan fuerte-

mente con otras moléculas pero no pueden ser transportados con eficiencia a través de la membreana. En cambio los cationes di-valentes pueden moverse a través de la membrana en forma relativa mente limitada, pero muestran un amplio rango de constantes de asociación con moléculas biológicas tanto en interfases membra-nales como en la fase acuosa; además los sitios de quelación ó los núcleos polares de polipéptidos y proteínas son más compati bles con el radio iónico del Ca^{2+} (1.0 Å) que con el del Mg^{2+} (0.7 Å)(Urry, 1978). Por estas razones podemos explicar porque los cationes monovalentes, como el Na^+ y el K^+, estan relaciona dos primariamente con corriente iónicas transmembranales, que son responsables por ejemplo de las propiedades bioeléctricas de las membranas excitables; y porque el Ca^{2+} se relaciona en primer lu gar con la modulación de las interacciones selectivas entre macro moléculas en el interior de la célula.

En tejidos excitables las funciones más ampliamente aceptadas para el Ca^{2+} se relacionan con el acoplamiento entre la excitación y la contracción en el músculo, y con el acopla-miento entre la excitación y la secreción en las terminales ner viosas y en algunas glándulas endócrinas. Para esto último se propone que la acción del Ca^{2+} se relaciona directamente con el mecanismo de la exocitosis de las vesículas que contienen el neurotransmisor o la hormona (Llinás y Heuser, 1977; Thorn et al., 1978).

Si bien para la placa neuromuscular existen evidencias que demuestran que la acetilcolina se libera de una poza de almacenamiento que se localiza en las vesículas sinápticas (Heuser y Reese, 1973; Heuser et al., 1979; Tauc, 1979); en el caso de los aminoácidos propuestos como neurotransmisores aún se desconoce el origen de la liberación espontánea y de aquella producida por despolarización, es decir que no siempre se relaciona con el contenido de las vesículas sinápticas. Existen algunos datos que sugieren que los aminoácidos neurotransmisores se liberan de una poza soluble o citoplásmica de la terminal sináptica (De Belleroche y Bradford, 1977). Para el ácido γ-aminobutírico (GABA), que es el neurotransmisor más ampliamente distribuido en el SNC de mamíferos (Krnjević, 1974; Curtis, 1975), se ha postulado la existencia de una liberación tónica (independiente de despolarización) que sería responsable de la regulación de la excitabilidad a través de una inhibición continua (Tapia,1974). Dicha liberación se llevaría a cabo a través del acoplamiento entre la síntesis y la liberación del GABA; pero el factor de acoplamiento también sería el Ca^{2+}, ya que se ha demostrado que la glutamato descarboxilasa (enzima responsable de la síntesis del GABA) es capaz de unirse, en presencia de Ca^{2+}, tanto a membranas sinaptosomales (Fonnum, 1968) como a membranas de fosfolípidos (Covarrubias y Tapia, 1978; 1980);

es decir que la enzima unida a la membrana sináptica por medio del Ca^{2+} estaría acoplada a un sistema que liberaría el GABA recién sintetizado, de tal manera que el Ca^{2+} bien podría ser el regulador de esta unión y por lo tanto también de la libera ción tónica de GABA. El mecanismo de liberación propuesto sería dependiente de Ca^{2+}, independiente de despolarización y no sería vesicular.

Con base en esta hipótesis, en la primera parte de esta tesis se estudiaron las características de la unión de la glu- tamato descarboxilasa a membranas de fosfolípidos, asi como las propiedades cinéticas de la enzima unida y de la enzima libre; encontrandose que la unión es de naturaleza primariamente iónica y que la enzima unida es más suceptible a la regulación de su actividad por la coenzima (fosfato de piridoxal).

Hemos propuesto que el acoplamiento entre síntesis y liberación de GABA podría ser un proceso regulado por $Ca^{2+}i$, sin embargo para poder correlacionar estos fenómenos sería impor tante cuantificar el $Ca^{2+}i$ en las terminales nerviosas aisladas. En la actualidad el estudio del $Ca^{2+}i$ como un regulador esta li mitado debido a que en ciertas preparaciones celulares (placa neuromuscular) y subcelulares (sinaptosomas) es difícil medir directamente los cambios en la concentración de este catión, puesto que por su tamaño no son accesibles a las técnicas electro

fisiológicas de inyección y iontoforesis. Son basicamente tres los métodos que se han utilizado para medir el $Ca^{2+}i$ en combina ción con técnicas electrofisiológicas (Ashley y Campbell, 1979):

1) Fotoproteínas sensibles a Ca^{2+} (Llinás y Nicholson, 1975).

2) Indicadores metalocrómicos sensibles a Ca^{2+} (Gorman y Thomas, 1978).

3) Microelectrodos sensibles a Ca^{2+} (Ashley et al., 1978).

Un buen método para cuantificar el $Ca^{2+}i$ deberá cubrir los siguientes criterios (Ashley y Campbell, 1979):

1) Sensibilidad y selectividad por el Ca^{2+}. El método deberá ser sensible al Ca^{2+} por lo menos hasta 0.1 uM ó 10 nM. Debido a que la concentración de Mg^{2+} en el citoplasma esta en tre 1 y 5 mM, el método deberá ser altamente selectivo por el Ca^{2+} en relación al Mg^{2+}.

2) Velocidad de la respuesta. El método deberá ser lo bastante rápido para detectar cambios en la concentración del $Ca^{2+}i$ en el rango de los milisegundos, ya que los eventos invo lucrados en la comunicación neuronal a corto plazo se llevan a cabo dentro de ese rango temporal.

3) Distribución. El método deberá permitir estudiar la distribución del Ca^{2+} en el citoplasma durante diferentes condi ciones experimentales.

4) Accesibilidad a las células. El método deberá permi

tir la penetración del indicador al interior de la célula.

5) Tolerancia biológica. El método no deberá causar daño a las propiedades fisiológicas y bioquímicas de la célula y no deberá alterar en forma importante el equilibrio del $Ca^{2+}i$.

Algunas de las preparaciones en las que se ha logrado medir directamente los cambios en la concentración de $Ca^{2+}i$ son: en músculo esquelético de rana y en fibras musculares de cangrejo (Miledi et al., 1977; Suárez-Kurtz, 1979); en axón gigante de calamar (Brown et al., 1975; DiPolo et al., 1976); en la célula R-15 del ganglio abdominal de Aplisia (Gorman y Thomas, 1978); y en los fotoreceptores ventrales de Limulus (Brown et al., 1977). Todas estas preparaciones permiten el uso de las técnicas electrofisiológicas de inyección.

En la segunda parte de esta tesis mi objetivo es demostrar que es posible medir cambios en la concentración de $Ca^{2+}i$ en sinaptosomas utilizando el indicador metalocrómico arsenazo III. Este colorante ha sido muy bien caracterizado y cubre los criterios anteriormente mencionados (Kendrick et al., 1977; Scarpa et al., 1978; Thomas, 1979). Esto permitirá correlacionar directamente un cambio en la concentración de $Ca^{2+}i$ con cualquier fenómeno fisiológico que a nivel molecular se supone esta regula- do por Ca^{2+} en las terminales nerviosas del SNC de mamíferos.

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Brain Glutamate Decarboxylase: Properties of Its Calcium-Dependent Binding to Liposomes and Kinetics of the Bound and the Free Enzyme

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Abstract: In the present work we describe several properties of the Ca^{2+}-dependent binding of glutamate decarboxylase (GAD) to phosphatidylcholine-phosphatidylserine liposomes. The binding occurs very rapidly, is dependent on temperature in the range 23–37°C, is inhibited up to 35% by K^+ in a concentration-dependent manner, and is slightly increased when the dielectric constant of the medium is decreased by 3% ethanol. The association of GAD and liposomes is very firm, since EGTA displaces only 40% of the bound enzyme, and Triton X-100 about 55%. Since apparently only part of the total GAD is able to bind to the liposomes, and in a previous study two forms of GAD activity have been identified kinetically, we compared the activations by pyridoxal 5'-phosphate (PLP) of the soluble and the bound GAD, as well as their inhibition by PLP oxime-O-acetic acid. The bound GAD was activated 150–265% by 10^{-6} to 10^{-4} M-PLP, whereas the activation of GAD that remained soluble was only 65–110% in the same PLP concentration range. In the absence of PLP, the bound GAD was less inhibited by the PLP oxime-O-acetic acid than the soluble GAD, but the inhibition was similar when 0.1 mM-PLP was added. In contrast, activity of both the soluble and the bound GAD was totally blocked by aminooxyacetic acid. Endogenous PLP did not bind to liposomes under the experimental conditions inducing GAD binding. We conclude that the binding of GAD to negatively charged liposomes is primarily ionic. Furthermore, the GAD molecules that bind to the liposomes seem to be deficient in free PLP and, therefore, are probably more susceptible to regulation by the coenzyme. These conclusions may be relevant to the hypothesis of a coupling between synthesis and release of GABA in inhibitory nerve endings. Key words: Glutamate decarboxylase—Liposomes—Calcium—Pyridoxal phosphate—Protein-phospholipid interaction.

We have postulated that brain glutamate decarboxylase (GAD; L-glutamate-1-carboxylyase, EC 4.1.1.15) plays an important role in the regulation of CNS excitability through the coupling of the synthesis and the release of GABA (Tapia, 1974; 1975; Tapia et al., 1975). Because this coupling must be a membrane phenomenon, the possibility that some form of GAD may bind to membranes is particularly relevant to this hypothesis. In a previous communi-

cation (Covarrubias and Tapia, 1978) we have shown that GAD present in a soluble preparation of brain tissue binds to phosphatidylcholine-phosphatidylserine multilamellar liposomes in a Ca^{2+}-dependent manner. The binding seems to be specific for GAD and does not occur with phosphatidylcholine liposomes lacking phosphatidylserine. The usefulness of this type of phospholipid vesicles as an experimental model for

studying the binding of GAD to membranes was made evident by the similarity of these results with those reported with synaptosomal membranes (Fonnum, 1968). In the present paper we report several physicochemical properties of the Ca^{2+}-dependent binding of GAD to phosphatidylcholine-phosphatidylserine liposomes, and we propose a mechanism for this binding.

Because apparently only part of the population of GAD molecules is capable of binding to liposomes, the possibility exists that this population is different from that which remains soluble (Covarrubias and Tapia, 1978). Therefore, the kinetic properties of GAD bound to liposomes were compared with those of the soluble enzyme.

The results obtained indicate that the binding mechanism is primarily ionic. Some differences in the activation by pyridoxal 5'-phosphate (PLP) and in the inhibition by pyridoxal phosphate oxime-*O*-acetic acid were found between the soluble GAD and the GAD bound to liposomes. These differences seem to be due to the preferential binding of GAD molecules devoid of PLP.

MATERIALS AND METHODS

Binding Experiments

The enzyme preparation, the preparation of liposomes, the binding incubation conditions, and the method used for GAD activity determination were all as previously described (Covarrubias and Tapia, 1978). Briefly, liposomes were obtained by shaking a 3% water suspension of phosphatidylcholine-phosphatidylserine mixture (4:1 by wt.) under nitrogen atmosphere. Liposomes and the enzyme preparation (100,000 g supernatant of mouse brain homogenates prepared in water containing 0.1 mM-PLP) were incubated in the presence of 2 mM-Ca^{2+} and 0.1 mM-PLP during various time periods, at 37°C, or at different temperatures for 20 min in a volume of 8.5 or 4.2 ml. The pH was 6.8–7.1, and the ratio of phospholipid to protein was 1.5 (w/w). The binding was stopped by centrifugation at 100,000 g for 30 min at 0–4°C. GAD activity was measured in the supernatant and in the liposomal pellet resuspended in 1–2 ml of 0.4% Triton X-100 containing 0.1 mM-PLP. The zero-time values were obtained by mixing the enzyme preparation and the liposomes at 0–1°C in a centrifuge tube and centrifuging immediately. Protein was determined by the method of Lowry et al. (1951).

In some experiments the ionic strength of the binding medium was increased by adding different concentrations of potassium acetate. In an effort to decrease the dielectric constant of the medium, ethanol was added in some experiments to give a 3% (v/v) final concentration. This ethanol concentration does not inhibit GAD activity (Wu and Roberts, 1974). The change in the dielectric constant was assessed by measuring at 37°C the conductivity of a Ca^{2+} aqueous solution containing 3% ethanol; a 14% decrease of the conductivity was observed.

Binding Stability

Some experiments were carried out in order to study the reversibility of the binding of GAD to liposomes. After binding as described above, in a volume of 4.2 ml, the stability of the binding was tested by resuspending the liposomal pellet in 4 ml of various solutions of 0.1 mM-PLP, 1–2 mM-EGTA, 0.2–1% Triton X-100, and a mixture of 2 mM-EGTA and 1% Triton. After a few minutes at 0–4°C the suspension was centrifuged again at 100,000 g for 30 min. The pellet was resuspended in 1–2 ml of 0.1 mM-PLP, and GAD activity was measured in the two supernatants, in the final pellet, and, in order to calculate the recovery, in an aliquot of the enzyme preparation. The average recovery in all experiments was 70%.

Kinetic Studies

In the presence of several concentrations of glutamate and PLP the activities of GAD were obtained both for the enzyme bound to the liposomes and for the soluble enzyme. In the experiments in which PLP concentration was varied, this coenzyme was omitted from all the media used for GAD preparation, binding incubation, and liposomal resuspension. The effect of aminooxyacetic acid and its PLP oxime (PLP oxime-*O*-acetic acid), which is an inhibitor acting exclusively on free PLP-dependent GAD activity (Tapia and Sandoval, 1971; Bayón et al., 1977a,b), was also assessed on both the liposome-bound and the soluble GAD, in both the absence and the presence of 0.1 mM-PLP. In all kinetic experiments the binding incubation volume was 8.5 ml, and the liposomal pellet was resuspended in enough water to obtain the necessary volume for measuring GAD activity simultaneously at the several glutamate or PLP concentrations used.

Binding of PLP to Liposomes

In one series of experiments the binding of endogenous PLP to liposomes was studied. The incubation conditions were as described above for GAD binding, except that exogenous PLP was never added to the media for enzyme preparation, binding incubation, or liposomal resuspension. Endogenous PLP concentration was measured in the enzyme preparation and in the liposomal supernatant after incubation by the apotryptophanase method of Wada et al. (1969), as modified by Minard (1967). Previous to PLP measurement, the samples were deproteinized with perchloric acid; the latter was removed by neutralization with KOH and subsequent centrifugation of the potassium perchlorate formed. The difference between the PLP content of the enzyme preparation and that of the liposomal supernatant represented the binding of PLP to liposomes.

Materials

L-α-Phosphatidylcholine from egg yolk (type V-E) and phosphatidylserine (bovine brain extract, type III, containing 80–85% phosphatidylserine, 5–10% cerebrosides, and 5% phosphatidic acid) used for liposome preparation were from Sigma Chemical Co. (St. Louis, Missouri). We have shown previously that the same GAD binding results are obtained when a pure phos-

phatidylserine is used (Covarrubias and Tapia, 1978).
PLP and *E. coli* tryptophanase (grade II) were also from
Sigma. Apotryptophanase was obtained by dialysis for
20–24 h of the tryptophanase against water at 0–4°C.
PLP oxime-*O*-acetic acid was synthesized from PLP and
aminooxyacetic acid as previously described (Tapia and
Sandoval, 1971). All other nonradioactive chemicals used
for assays were from Sigma. DL-[1-¹¹C]glutamic acid was
obtained from New England Nuclear (Boston, Mas-
sachusetts) or from the Radiochemical Centre (Amer-
sham, U.K.).

RESULTS

Binding Experiments

As seen in Fig. 1, the major portion of GAD
binding is very rapid. Immediately, 32% of the en-
zyme is bound to the liposomes, and this value in-
creases progressively to a maximum of about 45%
at 20 min. The binding is independent of tempera-
ture in the range 1–23°C, increases sharply between
23 and 28°C, and reaches an apparent maximum at
37°C (Fig. 2).

When the ionic strength of the medium was in-
creased with potassium acetate, the binding of GAD
was considerably blocked. The inhibition varied
from 15% at 12.5 or 25 mM-K⁺ to 32% with 100
mM-K⁺ (Fig. 3). In contrast, when the dielectric
constant was reduced by the addition of 3% ethanol,
a slight increase of GAD binding (21%, mean of
three experiments) was observed at a 2 mM-Ca⁺
concentration.

Dissociation of Bound GAD

In order to study the strength of the GAD-Ca²⁺-
liposome association, attempts were made to dis-

FIG. 2. Ca²⁺-dependent binding of GAD to liposomes as a
function of temperature. Liposomes were incubated with the
enzyme preparation for 20 min at the indicated temperatures.
Mean values of the number of experiments shown in par-
entheses ± S.E.M.

place the GAD bound to the liposomes. For this
purpose, we tested 0.1 mM-PLP, 1–2 mM-EGTA,
and 0.2–1% Triton X-100, as described in Methods.
Washing of the GAD-liposomes pellet with PLP
or EGTA resulted in a dissociation of 32% and
41%, respectively, of the GAD bound, whereas
Triton X-100 at any of the concentrations used
dissociated 56% of the bound enzyme (Table 1).
In one experiment, the addition of a mixture of
2 mM-EGTA and 1% Triton X-100 did not in-
crease the dissociation produced by the detergent
alone.

Kinetics of Bound GAD and Soluble GAD

In order to study the possibility that only one of
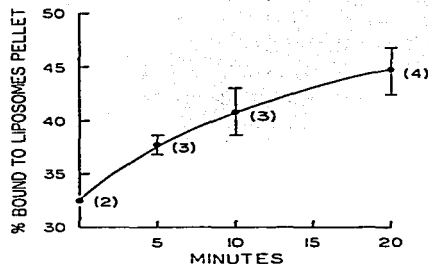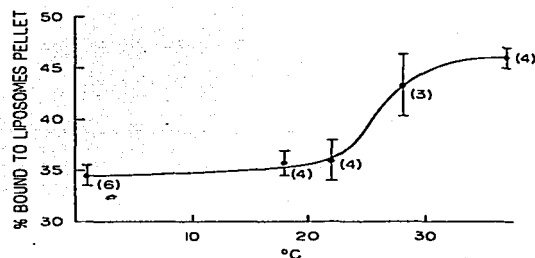the two forms of GAD which we have postulated

FIG. 1. Time course of the Ca²⁺-dependent binding of GAD to
phosphatidylcholine-phosphatidylserine liposomes. Lipo-
somes were incubated with the enzyme preparation at 37°C
in the presence of 2 mM-Ca²⁺ for the times indicated, and the
reaction was stopped by centrifugation, as described in Ma-
terials and Methods. The percent bound was obtained con-
sidering as 100% the value of the pellet plus that of the su-
pernatant. Mean values of the number of experiments shown
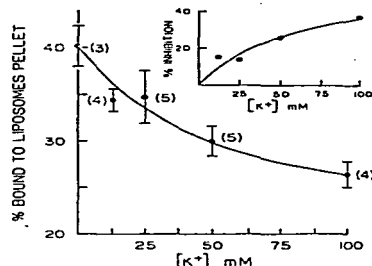in parentheses ± S.E.M.

FIG. 3. Effect of K⁺ on the Ca²⁺-dependent binding of GAD to
liposomes. Ca²⁺ (2 mM) and the indicated concentrations of
potassium acetate were present during 20 min incubation at
37°C of the liposomes with the enzyme preparation. Mean
values of the number of experiments shown in parentheses ±
S.E.M. In the inset, the percent inhibition of the binding is
plotted as a function of K⁺ concentration.

TABLE 1. *Dissociation of the GAD bound to liposomes*

Treatment of liposomal pellet	% of bound GAD released
PLP (0.1 mM)	31.6 ± 3.8 (3)
EGTA (1 or 2 mM)	41.0 ± 4.5 (6)
Triton X-100 (0.2 – 1%)"	55.8 ± 6.3 (6)

After binding incubation the liposomal pellet was resuspended in the indicated solution and centrifuged again, as described in Methods. Mean values of the number of experiments shown in parentheses ± s.e.m.

" Similar values were obtained with 0.2, 0.4, 0.6, and 1% Triton X-100.

(Tapia and Sandoval, 1971; Bayón et al., 1977b) is capable of binding, we compared the activity of the GAD bound to liposomes and that of the soluble GAD in the presence of different concentrations of PLP at a saturating glutamate concentration (20 mM) and in the presence of increasing glutamate concentrations at a saturating PLP concentration (0.1 mM). The endogenous PLP concentration in the GAD assay mixture (0.7×10^{-7} M, measured as described in Methods; see below) is negligible compared with the smallest PLP concentration used for obtaining the PLP curves (10^{-6} M); therefore, this value was not considered in the curves. As is shown in Fig. 4, the activation of GAD by PLP, expressed as percentage of the value in the absence of exogenous PLP, was much greater in the liposomal pellet
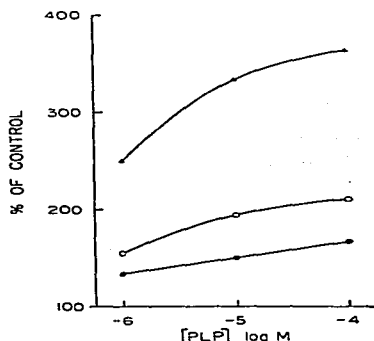

FIG. 4. Activation of the soluble GAD and of the GAD bound to liposomes by different concentrations of PLP. (O) GAD original preparation (100,000 g homogenate supernatant); (O) GAD in liposomal supernatant; (▲) GAD in liposomal pellet. No PLP was added to the enzyme preparation, the incubation medium, or the pellet resuspension medium. The control (100%) refers to the activity in the absence of exogenous PLP. Each point is the mean value of three experiments for 10^{-6} and 10^{-5} M-PLP and seven to nine for 10^{-4} M-PLP. The s.e.m. of the points varied from 1.3 to 5.2% of the corresponding value.

than in the liposomal supernatant or the original GAD preparation. Whereas the bound GAD was activated 150–265% by 10^{-6} to 10^{-4} M-PLP, the activation of the GAD that remained soluble was only 65–110% at the same PLP concentration range. The K_m for PLP was similar for the bound GAD, the soluble GAD, or the original homogenate supernatant (3.8×10^{-7} M, 4.0×10^{-7} M, and 1.3×10^{-7} M, respectively; Fig. 5). A slight, and probably insignificant, difference was found between the K_m of the bound GAD and the soluble GAD with respect to glutamate (5.6 mM and 3 mM, respectively; Fig. 6). The V_{max} of the GAD bound obtained in the PLP plot was about 2.76 times that of the soluble enzyme (Fig. 5). This is consistent with the difference observed in the glutamate plot, in which the V_{max} ratio bound/soluble was 3.0 (Fig. 6).

In an attempt to correlate the observed differences in the activation by PLP with the presence of free PLP-dependent and free PLP-independent forms of GAD activity, previously postulated on kinetic bases (Bayón et al., 1977b), we studied the inhibition of the liposome-bound and of the soluble enzyme by aminooxyacetic acid and PLP oxime-O-acetic acid, in both the absence and the presence of 10^{-4} M-PLP. Although the former compound inhibits both types of GAD activity, the latter appears to block only the free PLP-dependent activity (Tapia and Sandoval, 1971; Bayón et al., 1977a). Table 2 shows that with 10^{-4} M-PLP this PLP-oxime produced about the same degree of inhibition (66–70%) in the bound and the soluble GAD; but in the absence of added coenzyme, the soluble enzyme was considerably more inhibited than the bound enzyme (50% and 25%, respectively). In contrast, aminooxyacetic acid notably inhibited both enzyme preparations (Table 2).
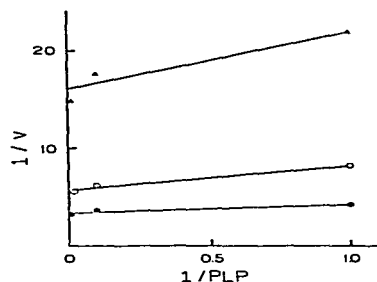

FIG. 5. Double reciprocal plot of GAD activity (v = c.p.m. × 10^{-3}/mg protein) as a function of PLP concentration (μM) in the original preparation (O), liposomal supernatant (▲), and liposomal pellet (O). Same experiments as those of Fig. 4. Mean values of three to nine experiments.
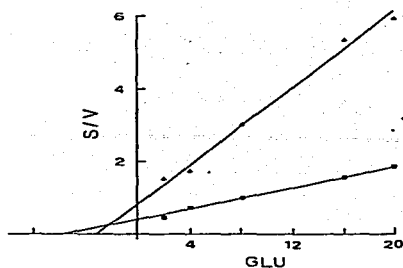
FIG. 6. GAD activity in liposomal supernatant (▲) and liposomal pellet (□) as a function of glutamate concentration: $S(mM)/v$ (c.p.m. $\times$ 10^{-3}/mg protein) vs S (mM). Mean values of two to three experiments.

Binding of PLP

The differences in the activation and inhibition between the GAD bound to liposomes and the soluble GAD, described above, could be accounted for by differences in the concentration of PLP in the liposomes pellet and supernatant. In order to test this possibility, we studied the binding of endogenous PLP to liposomes in the presence of Ca^{2+}, as described in Methods. The results of these experiments are shown in Fig. 7. It can be seen that all the endogenous PLP remains soluble after incubation under conditions identical to those of GAD binding. In other experiments the bound enzyme (in the absence of PLP) was dissociated from the liposomes with 0.7% Triton X-100, as described above, and its activation by 10^{-4} M PLP was studied. The activation was similar to that of the enzyme associated to the liposomes (224%; mean of two experiments).

DISCUSSION

Binding Characteristics

The sharp increase in GAD binding observed between 23°C and 37°C might be related to a ther-
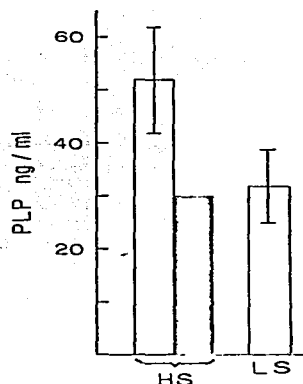

FIG. 7. Endogenous PLP concentration in the enzyme preparation (100,000 g homogenate supernatant—HS) and in the liposomal supernatant (LS) after incubation as for GAD binding, in the presence of 2 mM-Ca^{2+}. The open bars indicate the actual measurements, and the solid bar the value of PLP in HS after correction for the dilution of the liposomal supernatant, in order to make comparable the values of HS and LS. Mean values of four experiments $\pm$ S.E.M.

motropic transition of the phosphatidylcholine-phosphatidylserine membranes to a more fluid state (Tanford, 1973; Chapman, 1978), which favored the Ca^{2+}-dependent binding of GAD. Although we obviously do not know the length and saturation of the acyl moieties of the phospholipids used, the transition temperatures of similar phospholipid mixtures is in the same range as that in which the increase of GAD binding was observed (Tanford, 1973; Papahadjopoulos et al., 1974; Jacobson and Papahadjopoulos, 1975). Since the binding of GAD at zero time was very similar to that observed after 20 min at 2–20°C (approx. 33%; Figs. 1 and 2), it seems possible that below the transition tempera-

TABLE 2. *Inhibition of the soluble GAD and the GAD bound to liposomes by PLP oxime-O-acetic acid and by aminooxyacetic acid*

	Percent inhibition		
		Liposomes	
Inhibitor	Homogenate supernatant	supernatant	pellet
PLP oxime-*O*-acetic acid (1 mM)			
No PLP added	69.3 ± 3.68	49.9 ± 1.05	24.5 ± 0.42
With 0.1 mM-PLP	75.3 ± 1.95	66.4 ± 2.59	70.2 ± 2.28
Aminooxyacetic acid (no PLP added)			
0.01 mM	83.6	75.2	75.2
0.1 mM	98.7	95.4	92.3

Mean values of four experiments ± S.E.M. for PLP oxime-*O*-acetic acid; two experiments for aminooxyacetic acid.

ture a constant proportion of GAD binds instantaneously to the more "solid" liposomes. The facilitated binding to the more "fluid" liposomes, probably produced above the thermotropic transition, clearly proceeds more slowly (Fig. 1).

The finding that Ca^{2+}-dependent GAD binding decreases with increasing potassium concentrations (Fig. 3) suggests that the binding mechanism works through electrostatic interactions. This interpretation is supported by the stimulation of the binding produced by a decrease of the dielectric constant produced by ethanol (Kauzmann, 1959), particularly at 2 mM-Ca^{2+}, and is also in agreement with the strict requirement of phosphatidylserine for the Ca^{2+}-dependent binding (Covarrubias and Tapia, 1978). From these data we conclude that the interaction between the phospholipid vesicles, Ca^{2+}, and GAD involves either salt linkages forming a Ca^{2+} bridge between the negative charges of GAD (the native enzyme is anionic; see Wu, 1976) and those of phosphatidylserine, or neutralization of these charges by Ca^{2+} with subsequent association of GAD molecules with the liposomes. The former hypothesis seems more probable in view of the fact that Ca^{2+} induces segregation of phosphatidylserine molecules in phosphatidylcholine-phosphatidylserine membranes in a form similar to its promoting effect on GAD binding (Ohnishi and Ito, 1974; Covarrubias and Tapia, 1978).

The Ca^{2+}-dependent association of GAD to liposomes is very firm, as is shown by the observation that EGTA released only 40% of the GAD bound; the reversal of the binding was more effective when Triton X-100 was added, but even under these conditions only 55% of the bound enzyme was released (Table 1). Interestingly, these results, as well as those described in our previous work (Covarrubias and Tapia, 1978), are very similar to those reported for synaptosomal membranes (Fonnum, 1968), and suggest that the binding of GAD to the latter membranes involves the type of Ca^{2+}-phosphatidylserine interaction discussed above.

Kinetic Experiments

There is evidence in the literature that enzymes generally accepted as being soluble might function in their native physiological state associated with some intracellular membrane or to the plasma membrane, and, furthermore, that the microenvironment provided by the membranes might affect their activity (Wilson, 1968; McLaren and Packer, 1970; Katchalski et al., 1971; Solomon and Miller, 1976; Wooster and Wrigglesworth, 1976). Although subcellular fractionation studies of GAD indicate that this enzyme is mainly soluble in the nerve endings (Fonnum, 1968; Pérez de la Mora et al., 1973),

its direct visualization with the use of antibodies has shown that it is associated with synaptosomal membrane structures (Wood et al., 1976). It was, therefore, of interest to study some properties of the activity of the GAD associated with the liposomes.

From our previous work (Covarrubias and Tapia, 1978) it can be calculated that the ratio of bound to soluble GAD relative specific activities in the presence of 2 mM-Ca^{2+} is 2.67. This value is in excellent agreement with the ratios V_{max} of GAD bound/V_{max} of GAD soluble obtained in the PLP and glutamate plots (2.75 and 3.0, respectively). Therefore, the differences in V_{max} observed may be explained by the relative specificity of the GAD binding to liposomes with regard to other proteins.

As shown in Figs. 4–6, the most notable difference between the GAD bound and that which remained soluble was that the former was considerably more activated by PLP than the latter, although the K_m for the coenzyme was similar. This finding is in good agreement with the observation that the bound GAD was less inhibited by the PLP oxime-O-acetic acid than the soluble GAD in the absence of PLP, the inhibition being similar when 0.1 mM-PLP was added (Table 2), because it has been demonstrated that this inhibitor blocks the activity of a GAD form dependent on the availability of free PLP (Tapia and Sandoval, 1971; Bayón et al., 1977a,b). Therefore, from these results we conclude that the GAD molecules bound by the liposomes are deficient in PLP as compared with those which remain soluble. This conclusion is strongly supported by the results of the experiments of PLP binding to liposomes. As shown in Fig. 7, 100% of the PLP present in the enzyme preparation (homogenate supernatant) remained soluble after incubation under the conditions inducing GAD binding.

Since we have previously shown that only one population of GAD is capable of binding to liposomes (Covarrubias and Tapia, 1978), it seems reasonable to conclude that this population is represented by the GAD molecules deficient in PLP and therefore more susceptible to regulation by the free coenzyme. If this interpretation is correct, its implications for the physiological role of GAD in nerve endings might be important. As mentioned above, although GAD is generally considered an intraterminal soluble enzyme, immunohistochemical studies demonstrate that the enzyme is associated with intraterminal membranes, including the presynaptic membrane (Wood et al., 1976). Thus, it is conceivable that at least two populations of GAD, one soluble and the other bound to the presynaptic membrane and to other intraterminal membrane structures in a Ca^{2+}-dependent manner, may be present in the GABAergic nerve terminals. The enzyme associated with the presynaptic membrane might be responsible for the postulated synthesis-secretion coupling of GABA (Tapia, 1974; Tapia

et al., 1975): and, because of its dependence on free PLP, it would be inhibited when the concentration of PLP is decreased.

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CHANGES IN THE INTRASYNAPTOSOMAL IONIZED CALCIUM CONCENTRATION: MEASUREMENT USING THE METALLOCHROMIC DYE ARSENAZO III.

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According to the 'calcium hypothesis' for the depolarization—induced release of neurotransmitters, an influx of extracellular calcium induced by depolarization leads to an increase in intracellular calcium concentration $[Ca^{2+}]i$ which in turn triggers transmitter release (1). Direct evidence for this hypothesis has been obtained through the injection of calcium into the nerve terminal of the squid synapse (2), by fusion of liposomes containing calcium with motor nerve terminals (3) and by the use of some calcium-ionophores in the absence of depolarization (4,7). An increase in the $[Ca^{2+}]i$ accompanying depolarization and transmitter release, was detected also by means of the light emitted by the calcium-sensitive photoprotein aequorin injected into the terminal (5).

In nerve endings isolated from mammalian central nervous system (synaptosomes), it has been shown that several depolarization conditions induce an increase in ^{45}Ca influx (6), as well as an increase in the release of neurotransmitter (7), and that this release is blocked by drugs inhibiting calcium transport (8). However no direct evidence of changes in intrasynaptosomal calcium concentration is available, mainly because of the lack of appropriate techniques for measuring directly such $[Ca^{2+}]i$ changes. In the present comunication we describe a method for detecting $[Ca^{2+}]i$ changes in synaptosomes. Our method consist in loading

synaptosomes with the metallochromic dye arsenazo III (O-(1,8 dihydroxy-3,6-disulfonaphtylene-2,7-bisazo)-bisbenzenearsonic acid), by means of their fusion, promoted by La^{3+}, with liposomes previously loaded with the dye. In a number of other preparations it has been shown that liposomes can transfer their aqueous contents through the cell membrane (9). It has demostrated also in several biological preparations that microinjected arsenazo III is a suitable indicator of $[Ca^{2+}]i$ (10-13), and can be used to measure micromolar ionized Ca concentrations in physiological salt solutions (14). With this method we have observed an increase of $[Ca^{2+}]i$ in the presence of the Ca-ionophore A23187 or of veratridine, a depolarizing alkaloid.

As shown in Fig. 1A phosphatidylcholine-phosphatidylserine liposomes prepared by sonication in the presence of 19.3 mM arsenazo III, did in fact incorporate the dye, since the addition of 2 mM $CaCl_2$ to the medium produced a notable change in the 660-690 nm differential absorbance (A and B are independent and continuous traces). That this observation is due to the influx of Ca^{2+} into the liposomes and not to the presence of contaminating arsenazo III in the medium is indicated by: a) the relatively slow change (the relaxation time of arsenazo III-Ca interaction is in the range of msec.) (15); b) the effect of A23187, which

induced a very rapid influx of Ca^{2+} into the liposomes until an apparent equilibrium was reached; and c) the blocking effect of La^{3+} on the spontaneous influx of Ca^{2+}, although the influx induced by A23187 was not affected (Fig. 1B). Fig. 1 (A and B) shows also that the addition of veratridine by itself did not affect the spontaneous influx of Ca^{2+} into the liposomes, with or without La^{3+}.

The Fig. 2 shows results obtained with dye loaded synaptosomes. The presence of contaminating free arsenazo III in the medium is excluded by the fact that after Ca^{2+} addition there is no instantaneous change in the differential absorbance, even at very high sensitivity setting of the spectrophotometer. Synaptosomal membrane seems to be almost impermeable to Ca^{2+}, since upon the addition of 2 mM $CaCl_2$ there is only a slow and small influx of Ca^{2+}, not always observed (see the recordings of three independent experiments in the top left part of Fig. 2). However, when 7.5 uM veratridine was added, in all cases the influx of Ca^{2+} was clearly stimulated and, as expected, this stimulation was prevented by substitution of Na^+, in the medium by choline$^+$ (see the top right three recordings in Fig. 2). Both in the presence or absence of Na^+, the addition of A23187 produced a rapid influx of Ca^{2+} into the synaptosomes, followed by a slow

apparent decrease in $[Ca^{2+}]i$. When 1.2 mM sodium phosphate was added to the medium such decrease was prevented (results not shown) and an increase in the spontaneous influx of Ca^{2+} was also observed. This suggest that this decrease in $[Ca^{2+}]i$ is due to the uptake of Ca^{2+} by some intraterminal stores (16), because phosphate would be mantaining it inaccessible to Ca^{2+}-buffering systems. The observation that this apparent decrease of $[Ca^{2+}]i$ was not observed in liposomes (compare Figs. 1 and 2 after A23187) supports this conclusion.

In order to measure quantitatively the changes in $[Ca^{2+}]i$ produced by veratridine and by A23187, the differential absorbance changes (660–690 nm) of the dye–Ca complex were calibrated at 33°C in a buffer of the following composition (mM): KCl 100, NaCl 4, $MgCl_2$ 2 and HEPES 10 (pH 7.0), to simulate the intracellular ionic medium. We selected the pair 660–690 nm in order to minimize the effect of Mg^{2+} on the absorbance of arsenazo III–Ca complex (10, 13). At 0.20 mM of the dye, the total calcium was varied from 0–400 uM, in the presence of 0.264 mM EGTA for buffering Ca^{2+}. Using 26100 as molar extinction coefficient (1 cm path length) of the arsenazo III–Ca complex (14), we obtained a linear relation of differential absorbance changes as a function of ionized calcium. Assuming a 1:1 arsenazo III–Ca complexing we obtain an apparent

an apparent dissociation constant of 10.6 uM, which is in the
range of that obtained in other laboratories at various ionic
strengths (10, 14, 17). Recently it has been demostrated that
arsenazo III forms 2:1 complexes with Ca^{2+}, but the assumption
1:1 is valid for calibration calculations because the $\Delta[Ca^{2+}]$ for
$\Delta A = \Delta Amax/2$ has a value similar to the apparent dissociation
constant obtained under that assumption if the same dye
concentration is always used (17). According to our calibration
system, a $\Delta A660-690$ of 0.001 represented a $[Ca^{2+}]i$ of 0.131 uM.
Therefore, a change of approximately 0.198 uM in the $[Ca^{2+}]i$
occurs within one min. after the addition of 7.5 uM veratridine
to arsenazo III-loaded synaptosomes, whereas the $[Ca^{2+}]i$ change
after A23187 addition is approximately five times larger (Fig. 2).
In order to discard unspecific changes in absorbance we also
performed the synaptosomes-liposomes fusion exactly under the
same experimental conditions described in Fig 2 but in the
absence of arsenazo III, or without La^{3+} to avoid fusion. In this
case we did not detect any appreciable change in the absorbance
(at the maximum sensitivity used) when adding $CaCl_2$, veratridine
and A23187 to the cuvette.

In order to have an idea of the intrasynaptosomal
concentration of free arsenazo III, synaptosomes loaded with the

dye as described in Fig. 2 were sedimented by centrifugation and resuspended in Na-5K medium containing 0.4% Triton X-100. Immediately after the addition of the detergent, the suspension turned blue-green, possibly because of some contaminating La^{3+} that was used to induce fusion, which became accessible to the released arsenazo III. This suspension was centrifuged at 100,000 g for 30 min. and 1 mM EGTA was added to the supernatant in order to chelate La^{3+} and thus obtain free arsenazo III. By measuring the absorbance of this supernatant at 570 nm and following the assumption of Blaustein (6) for the calculation of the synaptosomes volume per mg protein, we concluded that the concentration of arsenazo III in the fused synaptosomes was within 0.4-0.6 mM. These relatively high concentrations of arsenazo III permit a high Ca^{2+} selectivity, but the Ca^{2+}-buffering capacity of the dye may be increased (17).

The results presented in this paper demonstrate that the fusion of synaptosomes with unilamellar liposomes prepared in the presence of high concentrations of arsenazo III is an adequate procedure for introducing the dye into isolated nerve terminals and that it can be used for measuring changes in their $\left[Ca^{2+}\right]i$. To our knowledge the present report constitute the first successful attempt to measure directly $\left[Ca^{2+}\right]i$ changes in nerve endings from mammalian central nervous system.

Acknowledgements.

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Fig. 1. Influx of Ca^{2+} into phosphatidylcholine-phosphatidylserine liposomes in a medium containing (mM): 135 NaCl; 5 KCl; 10 glucose; and 20 Tris-HCl, pH 7.4 (medium Na-5K). Thirty mg of egg phosphatidylcholine in chloroform-methanol (type V-E from Sigma Chemical Co. St Louis Mo.) was dried with N_2, and then mixed with 10 mg of dry phosphatidylserine (bovine brain extract, type III, containing 80-85% phosphatidylserine, 5-10% cerebrosides and 5% phosphatidic acid, from Sigma). This mixture was dispersed by vortexing in 1 ml of 0.32 M glucose containing 19.3 mM arsenazo III (grade I approximately 98% pure, from Sigma), sonicated at 0-4°C during 4 min. in a MSE sonicator and centrifuged at 100,000 g for 20-30 min. to eliminate multilamellar liposomes. Unilamellar liposomes (0.8-1.0 ml) were then filtered trough a Sephadex G-50 column (30 cm x 1 cm), previously equilibrated with 0.32 M glucose, in order to separete the arsenazo containing liposomes from the arsenazo free in the medium. The liposomes loaded with arsenazo III (about 3.0 ml) were obtained in the void volume. The influx of Ca^{2+} into this fraction (about 2.6 mg phospholipid in the assay) was studied by measuring the differential absorbance change at 660-690 nm in a 3 ml cuvette (1 cm path length) in an Aminco DW-2a spectrophotometer, at 33°C. This change represents the formation of the arsenazo III-Ca

complex. The symbols indicate the addition of the following
compounds and its final concentration in the cuvette: La^{3+},
0.13 uM $LaCl_3$; Ca^{2+}, 2 mM $CaCl_2$; V, 7.5 uM veratridine (Sigma);
A, 2 ug Ca-ionophore A23187 (in 2 ul of ethanol). Each group of
three recordings (A and B) represents successive additions of
the indacated compounds, but A and B are different experiments.
Fig. 2. Changes of $\left[Ca^{2+}\right]$i in synaptosomes. Synaptosomes from
mouse brain, prepared as described by Hajós (18), were fused
with the phosphatidylcholine-phosphatidylserine liposomes
containing arsenazo III, which were prepared as described in
the legend of Fig. 1. For the fusion, freshly prepared synaptosomes
(about 3 mg protein, resuspended in .1 ml of 0.32 M glucose)
were mixed with the arsenazo III-loaded liposomes (about 13 mg
phospholipid) in the presence of 400 uM $LaCl_3$ to induce fusion
(19). After 10 min incubation at 37°C the mixture was centrifuged
at 24000 g for 10 min to eliminate nonfused liposomes and the
excess of La^{3+}, which remain in the supernatant. The synaptosome-
liposome pellet was resuspended in 0.5 ml of the Na-5K medium,
or in choline-5K medium, in which the NaCl was substituted by
equimolar choline chloride concentration. To measure the changes
in $\left[Ca^{2+}\right]$i, 0.2 ml of the synaptosomes-liposomes fraction was
diluted with 2.8 ml of the same resuspension medium, equilibrated

at 33°C for 15-20 min and the changes in $\Delta A660-690$ were measured

as described in the legend of Fig. 1. The symbols for the

additions are as indicated for Fig. 1. Three independent experiments

for each medium are shown for veratridine effect, and two for the

effect of A23187.

Na 5K

Ca²⁺   V

0.001 ΔA
50 sec

A

0.004 ΔA
50 sec

CHOLINE 5K

Ca²⁺   V

A