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INSTITUTO DE INVESTIGACIONES BIOMÉDICAS
PROGRAMA DE DOCTORADO EN CIENCIAS BIOMÉDICAS

**FISIOLOGIA MOLECULAR DEL
COTRANSPORTE DE K^+ : Cl^-**

(COMPARACION ENTRE LOS MIEMBROS
DE LA SUBFAMILIA DE LOS KCCs)

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QUE PARA OBTENER EL GRADO DE:

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PAGINACION

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ING. LEOPOLDO SILVA GUTIERREZ

Director General de la
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Presente.

Por medio de la presente me permito informar a usted que en la reunión del Subcomité Académico del Doctorado en Ciencias Biomédicas que se llevó a cabo el día 7 de marzo del presente año, se acordó designar el siguiente jurado para examen de Doctorado en Ciencias Biomédicas de la Biol. ADRIANA PATRICIA MERCADO CABRERA, con no. de cuenta 88338697 y no. de expediente 30971125 con la tesis titulada: "Fisiología Molecular del Cotransporte de K⁺:Cl⁻", dirigida por el Dr. Gerardo Gamba Ayala.

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El presente trabajo se realizó en la Unidad de Fisiología Molecular del Instituto de Investigaciones Biomédicas de la UNAM, ubicada en el departamento de Nefrología y Metabolismo Mineral del INCMNSZ, bajo la asesoría del Dr. Gerardo Gamba Ayala y en colaboración con el Dr. David B. Mount de la Universidad de Harvard.

**A toda mi familia,
especialmente a mis
adorados enanitos,
Natalia, Astrid,
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INDICE

RESUMEN	i
ABSTRACT	ii
INTRODUCCION	1
REGULACION DEL VOLUMEN CELULAR	
Transporte de iones	3
Aumento regulador del volumen celular (ARVD)	4
Disminución reguladora del volumen celular (DRVC)	5
TRANSPORTE ELECTRONEUTRO DE K^+ y Cl^-	
El cotransportador de K^+:Cl^- en eritrocitos	7
El cotransportador de K^+:Cl^- en células no eritroides	16
Túbulo proximal	17
Asa de Henle	18
Túbulo distal	19
Otras posibles funciones del cotransportador de K^+:Cl^-	19
Biología molecular de los cotransportadores electroneutros acoplados a cloro (CCC)	20
Características moleculares de la subfamilia de los cotransportadores electroneutros de potasio-cloro	23
HIPOTESIS Y OBJETIVO	30
METODOLOGIA	
Identificación molecular de $KCC3$ y $KCC4$	31
Clonación del DNAC	31

Análisis de la secuencia de los DNAc de KCC3 y KCC4	33
Localización cromosómica y estructura genómica de las isoformas KCC3 y KCC4 en el humano	34
Extracción de RNA total	35
Análisis de northern-blot para hKCC3 y mKCC4	35
Transcripción <i>in vitro</i> de RNAc	36
Análisis de la expresión funcional de los cotransportadores electroneutros	36
Obtención, preparación e inyección de los ovocitos de <i>Xenopus laevis</i>	37
Ensayo de captación para medir la expresión funcional de los cotransportadores de K⁺:Cl⁻ en ovocitos	37
RESULTADOS Y DISCUSION	39
El cotransportador de K⁺:Cl⁻ de los ovocitos de <i>Xenopus laevis</i>	56
RESULTADOS Y DISCUSION	58
COMENTARIOS FINALES Y CONCLUSIONES	70
BIBLIOGRAFIA	73
APENDICES	91

RESUMEN

Los cotransportadores de K^+Cl^- (KCC) forman una subfamilia de la familia génica de los cotransportadores electroneutros cloro-catiónicos, que a su vez está integrada por dos subgrupos filogenéticos: KCC2 con KCC4 y KCC1 con KCC3. Las cuatro isoformas del cotransportador de K^+Cl^- (KCC1-4) difieren significativamente en la distribución de los sitios potenciales para fosforilación y en la secuencia de algunos segmentos transmembranales. Usando expresión heteróloga en ovocitos de *Xenopus* hemos estudiado las consecuencias funcionales de ésta heterogeneidad estructural. Los ovocitos no inyectados muestran una captación de $^{86}Rb^+$ dependiente de Cl^- que se activa por aumento de volumen celular (hipotonicidad). Esta captación casi no se observa cuando los ovocitos no inyectados se exponen a isotonicidad, a excepción de KCC2; mientras que la hipotonicidad induce una activación de 28, 8, 45 y 54 veces en KCC1, KCC2, KCC3 y KCC4, respectivamente. Inhibidores del transporte afectan de diferente manera a los KCCs, con el siguiente porcentaje de inhibición para KCC1, KCC2, KCC3 y KCC4: 2 mM furosemida (90, 97, 88 y 38 %), 2 mM bumetanida (50, 98, 87 y 10 %), 100 μ M DIDS (75, 124, 100 y ND %), 100 μ M DIOA (50, 66, 50 y 70 %) y 10 mM bario (78, 76, 63 y 58%). El análisis cinético de KCC1, KCC2, KCC3 y KCC4 reveló una K_m para K^+ externo de 25.5 ± 3.9 , 9.3 ± 1.0 , 51.9 ± 3.8 , 17.5 ± 2.7 mM y para Cl^- externo de 17.2 ± 8.3 , 6.8 ± 0.9 , 14.2 ± 3.1 , 16.1 ± 4.2 mM, respectivamente. Todas las isoformas en isotonicidad fueron activadas por NEM y en hipotonicidad fueron inhibidas por caliculina A. Por lo tanto, los KCCs muestran importantes diferencias en sus propiedades cinéticas, sensibilidad a volumen, sensibilidad a NEM y respuesta farmacológica.

ABSTRACT

The K⁺:Cl⁻ cotransporters (KCCs) form a subfamily of the electroneutral cation-chloride cotransporter gene family, and fall into two phylogenetic subgroups; KCC2 paired with KCC4, and KCC1 with KCC3. The four isoforms of the K⁺:Cl⁻ cotransporters (KCC1-4), differ significantly in the distribution of predicted phosphorylation sites and in the sequence of key transmembrane segments. We have used heterologous expression in *Xenopus* oocytes to study the functional consequence of this structural heterogeneity. Non-injected oocytes exhibit a Cl⁻-dependent ⁸⁶Rb⁺ uptake, with two-fold activation by cell swelling (hypotonicity). Almost no Cl⁻-dependent ⁸⁶Rb⁺ uptake was observed in KCC-injected oocytes under isotonic conditions, whereas hypotonicity induced 28, 8, 45 and 54-fold activation of KCC1 KCC2, KCC3 and KCC4, respectively. Transport inhibitors differentially affected the four isoforms, with the following percentage inhibition for KCC1, KCC2, KCC3 and KCC4: 2 mM furosemide (90, 97, 88 and 38 %), 2mM bumetanide (50, 98, 87 and 10 %), 100 μM DIDS (75, 124, 100 and ND %), 100 μM DIOA (50, 66, 50 and 70 %), 10 mM barium (78, 76, 63 and 58 %). Kinetic analysis of KCC1, KCC2, KCC3, and KCC4 revealed a Km for extracellular K⁺ of 25.5 ± 3.9, 9.3 ± 1.0, 51.9 ± 3.8, 17.5 ± 2.7 mM and for extracellular Cl⁻ of 17.2 ± 8.3, 6.8 ± 0.9, 14.2 ± 3.1, 16.1 ± 4.2 mM, respectively. The four isoforms under isotonicity were activated by NEM and under hipotonicity were inhibited by calyculin A. Therefore, the KCCs exhibit important differences in their kinetic properties, volume-sensitivity, NEM-sensitivity, and pharmacological response.

Introducción

Las células eucariotas regulan su volumen a corto plazo debido a que controlan el movimiento de solutos y agua dentro y fuera de la célula a través de una serie de sistemas de transporte que les permiten responder al aumento o la disminución del volumen celular después de ser expuestas a soluciones hipotónicas o hipertónicas, respectivamente. Muchos de éstos sistemas de transporte ya han sido identificados molecularmente. No obstante, debido a que los flujos netos de electrólitos son activados durante la regulación del volumen celular, es probable que estos se lleven a cabo a través de vías de transporte electroneutras aún no identificadas. Algunas de estas vías pueden ser cotransportadores de cationes/aniones, intercambiadores o canales acoplados e inclusive una mezcla de todos ellos. Por lo anterior, el objetivo del presente trabajo fue identificar y caracterizar funcionalmente a nuevos miembros de transportadores electroneutros involucrados en la regulación del volumen celular.

Como ya se mencionó, las células eucariotas están dotadas con mecanismos de transporte que les permiten responder efectivamente a cambios de presión osmótica y mantener el agua en equilibrio químico a través de la membrana plasmática. Las células acumulan proteínas, aminoácidos y metabolitos de carbohidratos para realizar sus funciones básicas, lo que da como resultado un balance entre el volumen celular y el medio iónico interno, críticos para el metabolismo celular normal, la integración celular en el organismo, así como de la supervivencia en general (1).

Los gradientes iónicos que se establecen a través de las membranas, pueden ser usados para dirigir el movimiento de solutos o para producir y

transmitir señales eléctricas en células nerviosas y musculares. Debido a que el movimiento de agua a través de la membrana celular depende del gradiente de presión osmótica entre el interior y el exterior de la célula, ésta se acompaña de cambios en la concentración de moléculas osmóticamente activas. La capacidad de regular el volumen celular es una propiedad fundamental de muchas células. La homeostasis del volumen celular conjunta una serie de eventos que regulan la función celular (2). En condiciones fisiológicas basales, el volumen celular se mantiene constante mediante un mecanismo de salida, a través del cual la presión osmótica que aumenta en el citoplasma por los solutos impermeables es contrarrestada por otro mecanismo que emplea gasto constante de energía metabólica (3).

Una gran variedad de funciones celulares dependen críticamente del volumen celular. De hecho, la estructura y función de macromoléculas tales como las enzimas son afectadas por la solvatación; de modo que la integridad de las membranas celulares y la arquitectura del citoesqueleto pueden verse afectadas por alteraciones excesivas del volumen celular. Por tal motivo, las células han desarrollado un gran número de mecanismos reguladores que sirven principalmente para mantener el volumen constante, lo que implica eliminar los gradientes osmóticos a través de la membrana. Cuando la osmolaridad del espacio intracelular aumenta, la célula incrementa su volumen en respuesta al influjo de agua y activa vías de liberación de moléculas osmóticamente activas y agua que producen la recuperación del volumen celular.

Los mecanismos de regulación del volumen celular son finamente controlados por una maquinaria compleja de señales celulares que son

disminución reguladora del volumen celular (DRVC), ocurren a través de la activación de mecanismos de transporte específicos en la membrana plasmática que median el flujo neto de moléculas osmóticamente activas y por lo tanto de agua (5). Cuando se encogen por debajo de su punto basal, las células incrementan su volumen captando iones (ARVC), en cambio, cuando se hinchan más allá de su punto basal, disminuyen su volumen por la liberación de iones (DRVC).

Aumento regulador del volumen celular (ARVC)

Una de las vías a través de las cuales las células consiguen recuperar su volumen cuando este ha disminuido previamente por exposición en un medio hipertónico son los transportadores electroneutros; principalmente el cotransportador de $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ sensible a bumetanida (CSB2 también llamado NKCC1) y los intercambiadores de Na^+/H^+ (NHE1) y $\text{Cl}^-/\text{HCO}_3^-$ que se muestran

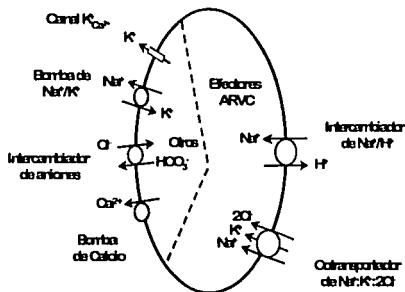


Figura 2. Mecanismos efectores del aumento regulador del volumen celular

en la figura 2. El CSB2/NKCC1 media la captación acoplada de Na^+ , K^+ y Cl^- y se encuentra virtualmente en todas las células (6). El NHE1 es una isoforma ubicua, que paralelamente con el intercambiador de $\text{Cl}^-/\text{HCO}_3^-$, están involucrados en la

captación de iones. El efecto combinado de ambos intercambiadores es la pérdida de H^+ y HCO_3^- y la consiguiente ganancia de NaCl . La bomba de

Na^+/K^+ es necesaria para el ARVC, debido a que intercambia Na^+ por K^+ y provee la energía suficiente para mantener bajas las concentraciones de Na^+ e indirectamente afecta la concentración de Cl^- intracelular ($[\text{Cl}^-]_i$). La disminución del volumen celular permite inhibir canales de K^+ y de Cl^- , minimizando así, la pérdida de KCl (5).

El mecanismo por el cual la disminución del volumen celular activa a CSB2/NKCC1 o NHE1 es desconocido. Debido a que la activación sucede en minutos, es poco probable que se deba a un incremento en la síntesis de proteínas. Se ha sugerido, sin embargo, que la fosforilación pudiera estar involucrada (5).

Otro mecanismo a través del cual las células pueden regular su volumen reduciendo el estrés osmótico sin afectar otras funciones celulares es la producción y acumulación de osmolitos orgánicos. En general las células de mamífero utilizan tres grupos de osmolitos: los polialcoholes (sorbitol e inositol), las metilaminas (betaina) y, los aminoácidos y sus derivados (glicina, glutamina, aspartato y taurina) (2). Gracias a estos compuestos es posible reemplazar altas concentraciones de sales intracelulares que podrían perturbar la estructura proteica y la función enzimática, lo que permite a células, como las de la médula renal, funcionar en altas osmolaridades (4).

Disminución reguladora del volumen celular (DRVC)

La figura 3 muestra los mecanismos que las células emplean para lograr disminuir el volumen celular cuando este ha aumentado previamente como consecuencia de su exposición a un medio hipotónico. Ésta disminución se lleva a cabo en parte por el flujo de K^+ y Cl^- a través de la activación de canales de K^+ y canales aniónicos. Los canales de K^+ son selectivos a cationes

monovalentes, mientras que los canales permeables a aniones permiten el paso de Cl^- , HCO_3^- y aún sustancias orgánicas tales como taurina y otros aminoácidos posiblemente cargados negativamente. Una amplia variedad de canales de K^+ y Cl^- activados por hinchamiento son utilizados para regular el volumen celular.

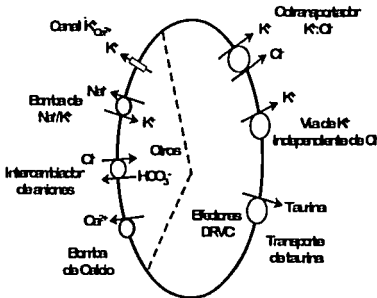


Figura 3. Mecanismos efectores de la disminución reguladora del volumen celular

Otro sistema de transporte que se muestra en la figura 3 y media la DRVC es el cotransportador de $\text{K}^+:\text{Cl}^-$, también conocido como KCC, que transporta K^+ y Cl^- con una estequiometría de 1:1 en cualquier dirección a través de la membrana plasmática.

Aunque estructuralmente está

relacionado con el cotransportador de $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ sensible a la bumetanida (CSB/NKCC) y es inhibido por la furosemida, este transportador se distingue por su independencia al Na^+ y su baja sensibilidad a la bumetanida (7). Entre los aniones fisiológicos, el cotransportador de $\text{K}^+:\text{Cl}^-$ tiene un estricto requerimiento por Cl^- y es uno de los principales mecanismos para la DRVC en células con una alta $[\text{Cl}^-]$ como los eritrocitos, en cuyo caso se ha propuesto que su activación está dada por la inhibición de proteínas cinasas. Debido a la falta de un inhibidor específico, el estudio del cotransportador de $\text{K}^+:\text{Cl}^-$ no ha sido fácil. Sin embargo, la abundancia de los RNA mensajeros (RNAm) sugieren que este mecanismo de transporte es más frecuente de lo que se

pensaba. La figura 4 es un esquema que ilustra las principales vías de transporte de solutos de la membrana celular de vertebrados. Como puede observarse, la célula debe estar dotada de todos aquellos mecanismos que le permitan responder adecuadamente a los cambios de volumen celular, dentro de los cuales se encuentra el cotransportador de K^+Cl^- .

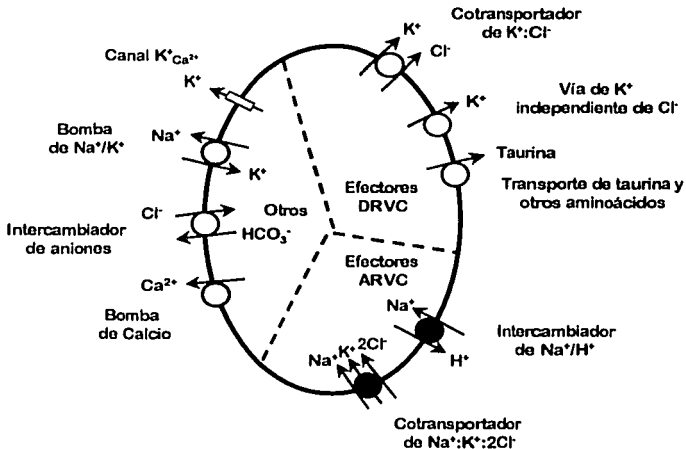


Figura 4. Diagrama esquemático que ilustra las principales vías de transporte a través de la membrana celular de vertebrados.

Transporte electroneutro de K^+ y Cl^-

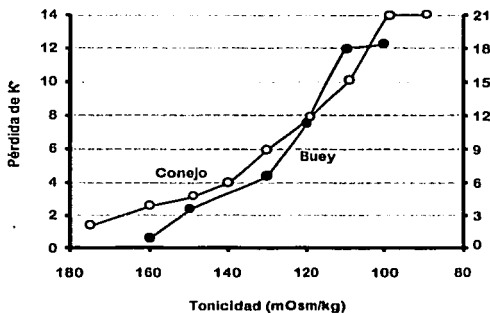
El cotransportador de K^+Cl^- en eritrocitos

Los cotransportadores de K^+Cl^- son proteínas de membrana que se conocen como cotransportadores electroneutros porque cada catión que acarrea de un lado a otro de la membrana celular va acompañado de un anión, sin producir cambios en el potencial transmembranal. En condiciones

fisiológicas el potencial químico para el K^+ promueve que la función del KCC sea una vía de eflujo de K^+ y Cl^- que dirige ambos iones hacia el exterior de la célula. La bomba de Na^+/K^+ a su vez contribuye a mantener dicho potencial químico.

El cotransportador de $K^+:Cl^-$ es un transportador de iones activo secundario debido a que utiliza los gradientes establecidos por el transporte activo primario a través de la bomba de Na^+/K^+ . Aunque las principales especies iónicas transportadas por el KCC son el K^+ y el Cl^- , se ha demostrado que el Rb^+ puede tomar el lugar del K^+ (8).

El cotransporte electroneutro de $K^+:Cl^-$ juega un papel fisiológico importante en numerosos tejidos, especialmente el riñón y el cerebro. Éste tipo de cotransporte fue originalmente descrito en eritrocitos como un mecanismo de eflujo de K^+ insensible a ouabaina, dependiente de Cl^- y activado por aumento del volumen celular. La figura 5 muestra uno de los primeros estudios,

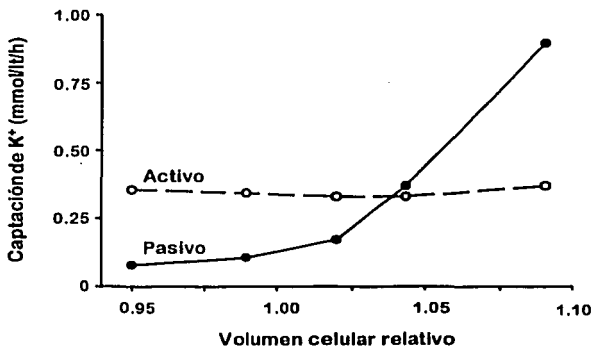


Davson H. 1937 *J Cell Comp Physiol*

Figura 5. Los eritrocitos de conejo y de buey son sensibles a la pérdida de potasio conforme disminuye la tonicidad del medio externo.

realizados en 1937 por Davson, donde observó que en eritrocitos de conejo y de buey se incrementa la permeabilidad del K^+ cuando las células se hinchan osmóticamente (9). Efectos similares

han sido demostrados para eritrocitos de perro y pato (10, 11) y también en otros tipos celulares (12-14). Sin embargo, no es sino hasta 1981 cuando Dunham y Ellory demostraron, en los eritrocitos de oveja de bajo K^+ (LK), la presencia de un flujo de K^+ insensible a ouabaina que es sensible a cambios del volumen celular y dependiente de Cl^- . La figura 6 muestra la relación entre el influjo pasivo de K^+ en eritrocitos de oveja LK y el volumen de las células. Cuando aumenta en un 10% el volumen celular la captación pasiva de K^+ en los eritrocitos se incrementa significativamente (8 veces). En cambio, cuando las células disminuyen ~ 5% de su volumen, dicha captación se reduce.



Dunham y Ellory 1981 *J Physiol*

Figura 6. Efecto de la variación del volumen celular sobre la captación pasiva y activa de K^+ en eritrocitos de oveja LK.

En el mismo experimento puede observarse que la captación activa de K^+ sensible a ouabaina permaneció casi constante en el rango de volúmenes estudiado. Es importante señalar que aunque el transportador funciona como una vía de eflujo de iones, es decir, hacia fuera, el invertir el gradiente del

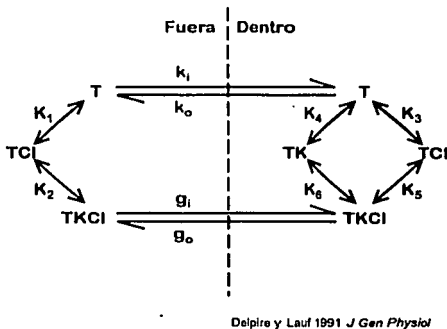
transporte resulta más sencillo de evaluar. De hecho, durante los primeros minutos después del aumento de volumen, ~ 2% de la radioactividad intracelular sale de la célula y aún en el medio hipotónico puede causar un sobreestimado significativo del eflujo inicial.

Los eritrocitos de oveja son un modelo interesante para estos estudios ya que muestran un dimorfismo del tipo K, en el cual se presentan dos fenotipos: uno de alta concentración de K intracelular (HK) y otro de baja (LK). Un antígeno del grupo sanguíneo designado L está asociado específicamente con el fenotipo LK y se ha demostrado que la sensibilización de células LK con el anticuerpo anti-L incrementan la captación de K^+ a través de la bomba de Na^+/K^+ (15) y disminuyen la captación de K^+ del componente insensible a ouabaína (16, 17). Este flujo de K^+ mostró ser altamente sensible a variaciones en el volumen celular, saturable a $[K^+]$ externo y dependiente de Cl^- , aunque el reemplazamiento de Cl^- por Br^- causó un incremento en la captación de K^+ (18).

Las mediciones cinéticas que en 1989 Brugnara y colaboradores realizaron del cotransporte de $K^+:Cl^-$ en eritrocitos humanos con Rb^+ , mostraron el efecto de cambiar el gradiente electroquímico del Cl^- sobre el movimiento neto de K^+ en presencia de un gradiente electroquímico constante de K^+ . Los resultados indicaron que los eritrocitos humanos, de conejo así como los de ovejas LK presentan un gradiente de Cl^- dirigido hacia afuera de la célula que conduce un eflujo neto de K^+ contra un gradiente electroquímico para K^+ dirigido hacia adentro, lo que promueve un transporte acoplado de K^+ y Cl^- que no afecta el potencial de membrana (19).

Lytle y McManus mostraron que el eflujo de K^+ y de Cl^- en eritrocitos de pato hinchados por exposición a un medio hipotónico es mutuamente

interdependiente y que una fracción del flujo de $^{36}\text{Cl}^-$ de los eritrocitos tratados con DIDS (ácido diisotiocyanatostilbene 2,2'-disulfónico) se inhibe por bumetanida y depende de la presencia de K^+ interno. En conjunto estos datos sugirieron que la disminución reguladora del volumen celular de eritrocitos hinchados osmóticamente está mediada por un sistema de cotransporte de $\text{K}^+:\text{Cl}^-$ (20). Más tarde se demostró que el coeficiente de Hill para este sistema tiene una estequiometría de 1:1, lo que indica que es electroneutro. El diagrama cinético de la figura 7 propuesto por Jennings (21), a partir de estudios con $^{36}\text{Cl}^-$, confirma que la estequiometría del transportador de $\text{K}^+:\text{Cl}^-$ es de 1:1, con una asimetría en la unión a iones que muestra en el exterior que la unión del Cl^- precede a la del K^+ mientras que en la cara interna ambos iones se unen azarosamente. La ventaja de ésta asimetría subyace en la posibilidad de que el K^+ externo module la afinidad del Cl^- con una V_{max} que llega a ser independiente de la concentración externa de cloro ($[\text{Cl}^-]_o$), mientras que la V_{max} está en función de $[\text{Cl}^-]_i$.



Delpire y Lauf 1991 *J Gen Physiol*

Figura 7. Esquema cinético del orden de unión a iones en el cotransportador de $\text{K}^+:\text{Cl}^-$ activado por aumento de volumen.

Los análisis de sustitución de aniones indican que el Br^- puede sustituir al Cl^- en eritrocitos de humano y ovejas. La selectividad relativa en eritrocitos de oveja sigue la serie $\text{Br}^- > \text{Cl}^- > \text{I}^- > \text{SCN}^- > \text{NO}_3^-$ (7, 22, 23). Actualmente no

existen inhibidores específicos para estudiar el cotransportador de K^+Cl^- . De hecho, todos los inhibidores estudiados hasta la fecha se unen o afectan a otros transportadores de membrana, como el intercambiador de Cl^-/HCO_3^- y al CSB/NKCC. Entre las drogas que inhiben el transporte de K^+Cl^- en eritrocitos se encuentran los llamados "diuréticos de asa" derivados del ácido sulfamoilbenzónico como la furosemida con un IC_{50} de ~ 0.5 mM que puede disminuir hasta 0.05 mM en presencia de K^+ externo. El efecto de la furosemida se logra a dosis menores que para el cotransporte de $Na^+K^+2Cl^-$ (CSB/NKCC) (7). El alcaloide DIOA (ácido dihydroindeyl-oxy alcalónico) es otro inhibidor que puede usarse con cautela para diferenciar entre el cotransporte de K^+Cl^- , el cotransporte de $Na^+K^+2Cl^-$ y otras vías de transporte aniónico; para el KCC en los eritrocitos su IC_{50} es cerca de 0.01 mM (24). Los derivados de los estilbenos disulfonados también inhiben, con menor especificidad, al cotransportador de K^+Cl^- . El efecto inhibitorio de dichos compuestos se probó en eritrocitos de ovejas del tipo LK sometidos a aumento de volumen por exposición a un medio hipotónico. De los estilbenos usados el DIDS mostró ser el de mayor potencia, con un IC_{50} para el cotransporte de K^+Cl^- de 60 μM y de 3 μM en ausencia y presencia de K^+ externo, respectivamente. Algunos otros derivados de los estilbenos como el SITS (ácido 4-acetamido-4'isothiocyanatostilbene-2,2'-disulfónico), el DNDS (ácido 4,4'-dinitrostilbene-2,2'-disulfónico) y el ISA (4-sulfophenyl isothiocyanate) mostraron poco efecto sobre el transportador a altas concentraciones (25). La quinina y la quinidina, otro tipo de drogas empleadas como bloqueadores de canales iónicos y que tradicionalmente sirven para bloquear canales de K^+ activados por Ca^{2+} , también inhiben al KCC (26) (Ver tabla 1).

Tabla 1. Inhibidores del cotransportador de K^+Cl^- en los eritrocitos

COMPUESTOS	IC ₅₀ (μM)	SITIO DE ACCION	ESPECIE
Furosemida	50 con $[K^+]_o$	Exterior del transportador	Oveja
DIOA	10	Exterior del transportador	Humano
DIDS	3 con $[K^+]_o$	Exterior del transportador	Oveja
Quinina	230	Interior del transportador?	Oveja
Protones	0.5	Mecanismo interno	Oveja, humano
Ácido okadaico	0.028, 0.04	PP1A, PP2A	Oveja, conejo
Calliculina A	0.005	PP1A	Todas
Genisteina	15	Transportador o tirosina cinasa?	Oveja
NEM	2000	Transportador	Oveja

En eritrocitos se ha demostrado que algunos de los activadores fisiológicos más importantes del KCC incluyen el aumento de volumen celular (27), la disminución del pH (28) y altas presiones parciales de O₂ (29). Entre los activadores farmacológicos del KCC se encuentran el agente alquilante de grupos sulfhidrido N-etilmaleimida (NEM), un compuesto pequeño, no polar y sin carga que penetra rápidamente la membrana celular (7, 30) y agentes oxidantes como la hidroxilamina (31), el peróxido de hidrógeno (32) y la diamida (33). Se considera que estos activadores actúan sobre los grupos sulfhidrido presentes en las proteínas reguladoras que se encuentran *cuesta arriba* en la cascada de activación (cinasas, fosfatasa, etc) (34, 35). Los activadores incrementan la V_{max} sin alterar las constantes de afinidad por los iones individuales (36) (Ver tabla 2).

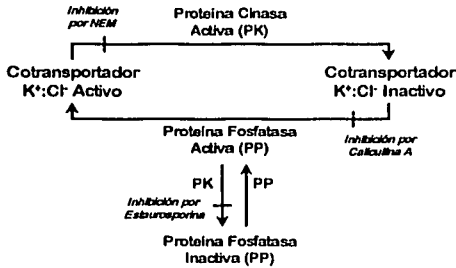
Tabla 2. Activadores del cotransportador de K^+Cl^- en los eritrocitos

COMPUESTOS	AC ₅₀ (μM)	SITIO DE ACCION	ESPECIE
NEM	0.2	Cinasas tioles	Varias
Diamida	0.2	GSH, otros tioles	Varias
H ₂ O ₂	2.1	Tioles, otros	Oveja, humano
NH ₂ OH	0.1	Hemoglobina?	Oveja
O ₂	-30torr	Hemoglobina, grupo hemo?	Varias
P _{O_{2m}}	n.d.	Acarreadores y cinasas	Varias
Estauosporina	<0.001	S/Th y Tyr cinasas	Oveja, humano

Existen datos que sugieren que el cotransporte de K^+Cl^- está regulado por un mecanismo complejo de fosforilación y desfosforilación. Para examinar el posible papel de las fosfatasa en la activación del cotransporte de K^+Cl^- , Jennings y Schulz (37), usaron ácido okadaico, un ácido graso de 38 átomos de carbono aislado originalmente de esponjas marinas que es un potente inhibidor de algunas proteínas fosfatasa. A concentraciones submicromolares el ácido okadaico inhibe el cotransporte de K^+Cl^- en eritrocitos de conejo activados por aumento de volumen celular (38). Ésta inhibición ocurre en un rango de concentración similar a aquel en que se inhibe a las proteínas fosfatasa del tipo 1 (PP1) (37).

El tratamiento de eritrocitos con NEM en medio isotónico activa al mismo sistema de cotransporte de K^+Cl^- que es activado por aumento del volumen celular. Sin embargo la relación entre las vías de activación por NEM o por hinchamiento no están claras. El efecto del NEM se complica debido a que el cotransportador de K^+Cl^- presenta al menos dos grupos sulfhidrilo con diferentes propiedades químicas (39, 40). El NEM por lo tanto puede afectar directamente al cotransportador modificando alguno de los grupos sulfhidrilo (40, 41), o puede actuar activando o inhibiendo enzimas que controlan la activación propia del transportador, concretamente a las proteínas cinasa. De este modo, la hipótesis más simple que Jennings propuso es que el aumento de volumen celular y el NEM causan la inhibición de una proteína cinasa que a su vez provoca la desfosforilación neta de algún sustrato clave (no necesariamente el transportador) y que el sustrato desfosforilado, probablemente la proteína fosfatasa 1 (PP1), por consecuencia, causa la activación del transportador. Así pues, la activación del cotransportador de

K^+Cl^- por aumento de volumen celular o NEM puede bloquearse por inhibidores de proteínas fosfatasa como caliculina A y/o ácido okadaico, lo que indica que la desfosforilación es necesaria para la activación. Además, inhibidores de proteínas cinasas como la estaurosporina, activan considerablemente al transportador (34, 38, 42-44). El actual modelo para la activación del cotransportador de K^+Cl^- se muestra en la figura 8 y sugiere que el transportador puede encontrarse en dos estados principales, activo (desfosforilado) o inactivo (fosforilado) (45, 46). Es posible que el balance entre



Cossins A & Gibson J 1997 *J Exp Biol*

Figura 8. Esquema propuesto para la fosforilación de una proteína que influye la actividad del cotransportador de K^+Cl^- en eritrocitos.

produce la activación que es catalizada probablemente por PP1. Sin embargo, las tirosinas cinasas también podrían jugar un papel importante en la regulación del transportador. Sachs (47) y Bize (48) mostraron, de manera independiente, que la genisteina, un inhibidor de tirosinas cinasas, inhibe el transporte de K^+Cl^- en eritrocitos humanos y de ovejas, respectivamente. Para confirmar lo anterior, Flatman y colaboradores (49) estudiaron el efecto de la genisteina

estos dos estados esté regulado por una cinasa que fosforile un residuo de serina o de treonina en una proteína (que incluso puede ser el transportador mismo) a favor de la forma inactiva, mientras que la reacción inversa

sobre la activación del cotransportador de K^+Cl^- en los eritrocitos de oveja por NEM y estaurosporina. La genisteína inhibió cerca del 90% del flujo neto de K^+ resistente a ouabaína de manera dependiente de la dosis ($K_i = 15 \mu M$), el transporte de K^+ dependiente de Cl^- y previno la activación del cotransportador después de un pretratamiento con 1 mM de NEM o 2 μM de estaurosporina. La constante de inhibición obtenida se asocia con su acción sobre una tirosina cinasa, por lo que sugirieron que la genisteína se une e inhibe al transportador o que la genisteína puede inhibir al transportador a través de la inhibición de una tirosina cinasa.

El cotransportador de K^+Cl^- en células no eritroides

Una gran cantidad de células exhiben sistemas de transporte de solutos que son influenciados por cambios en el volumen celular. En general, los flujos sensibles a cambios de volumen provocan que el volumen celular regrese a valores normales después de un aumento o disminución brusco.

Poco se conoce acerca de los mecanismos por los cuales un cambio en el volumen celular activa o inactiva ciertos transportadores. La primera información disponible a este respecto viene de estudios en los que el transportador es activado en ausencia de cambios de volumen. Por ejemplo, el cotransportador de $Na^+K^+2Cl^-$ en eritrocitos de aves puede ser activado por encogimiento celular o por catecolaminas (50), mientras que el intercambiador de Na^+/H^+ sensible a amilorida es activado por encogimiento celular, factores de crecimiento o por pH intracelular bajo (51). El cotransporte de K^+Cl^- independiente de Na^+ es activado por hinchamiento celular, por una ligera acidificación intracelular o por tratamiento con NEM (45, 52). Sin embargo, la característica de electroneutralidad y la falta de inhibidores específicos de alta

afinidad han dificultado el estudio del cotransportador de K^+Cl^- fuera del eritrocito. No obstante, el cotransportador de K^+Cl^- se ha reportado en un gran número de tejidos y tipos celulares incluyendo neuronas, músculo liso vascular, endotelio, epitelio, corazón y músculo esquelético (53-59). En consecuencia, el cotransportador de K^+Cl^- ha sido implicado no sólo en la disminución reguladora del volumen celular sino también en la absorción de sal transepitelial (57), la secreción renal de K^+ (60), la pérdida de K^+ durante la isquemia del miocardio (58) y la regulación de la concentración de Cl^- intraneuronal (53).

Poco después de su descripción en eritrocitos, se reportó la existencia de un cotransporte de K^+Cl^- en la membrana basolateral de células epiteliales del túbulo proximal y en la vesícula biliar de especies de *Necturus* (61, 62). Este transportador, además de funcionar como una vía de salida para K^+ y Cl^- , parece también participar en el reciclaje de K^+ hacia la membrana basolateral en los epitelios que reabsorben $NaCl$ (63). Estos y otros datos reportados en la literatura sugirieron la existencia del cotransportador de K^+Cl^- en el riñón.

Túbulo proximal

En vesículas de membrana basolateral de corteza renal de mamífero se detectó la actividad de un cotransporte de K^+Cl^- independiente de Na^+ (64). Igualmente existe evidencia fisiológica de la existencia del cotransportador electroneutro de K^+Cl^- en la membrana basolateral de los túbulos proximales (65, 66). En suspensiones de túbulos proximales de conejo, la activación del transportador de Na^+ :glucosa activa una vía de flujo de K^+ resistente a 5 mM Ba^{2+} y sensible a 1 mM de furosemida (67). Estos datos sugieren en conjunto que el cotransportador de K^+Cl^- de la membrana basolateral se activa por

aumento de volumen celular y juega un papel importante en la absorción transepitelial de sodio en el túbulo proximal. Ésta hipótesis es compatible con estudios de microperfusión realizados en rata donde se muestra que la furosemida intravenosa disminuye directamente la reabsorción de sodio en el túbulo proximal (68). Aunque se ha demostrado que el cotransportador de K^+Cl^- es una de las principales vías de salida de K^+ y Cl^- en la membrana basolateral del túbulo proximal, no es la única (69). El aumento de volumen celular también puede activar otra serie de proteínas como canales de K^+ y de Cl^- (70-72).

Asa de Henle

En 1983 Greger y Schlatter mostraron, por estudios electrofisiológicos en asas de Henle aisladas y perfundidas de riñón de conejo que un aumento en la $[K^+]_o$ despolariza la membrana basolateral y que esta despolarización podía inhibirse por Ba^{2+} . Con estos estudios concluyeron que la salida de K^+ por la membrana basolateral ocurre principalmente a través de un mecanismo de transporte electroneutro, donde el Cl^- sale de la célula acompañado por K^+ , o a través de canales conductivos (56). Éste transportador probablemente participa en la reabsorción transepitelial de sal en el asa de Henle, proporcionando una vía basolateral de salida de Cl^- y participando en el reciclaje de K^+ a través de la membrana basolateral. En la membrana apical del asa de Henle, el ion amonio (NH_4^+) puede sustituir al K^+ en el cotransportador de $Na^+K^+2Cl^-$. De hecho esta vía funciona en el mecanismo de contracorriente de NH_4^+ en el asa de Henle y se regula positivamente durante la acidosis metabólica (73). Trabajando con vesículas de membrana basolateral de asa de Henle de rata, Amlal y colaboradores encontraron un cotransportador de $K^+(NH_4^+):Cl^-$

susceptible de ser inhibido por altas concentraciones de furosemida (57). Esto sugiere que un cotransportador de K^+Cl^- renal también está involucrado en el reciclaje de NH_4^+ y secreción ácida renal.

Túbulo distal

La secreción de Cl^- acoplada a la del K^+ sugiere la presencia de un cotransportador apical de K^+Cl^- en el túbulo distal (74). En 1998 Weinstein predijo, mediante modelos matemáticos, la existencia de un cotransportador basolateral de K^+Cl^- en el túbulo colector medular interno (75). Actualmente, por estudios de inmunohistoquímica (76) se ha determinado que en los segmentos distales de la nefrona se expresan algunas de las isoformas del cotransportador de K^+Cl^- .

Otras posibles funciones del cotransportador de K^+Cl^-

Además del túbulo proximal y la porción gruesa del asa ascendente de Henle, el cotransportador de K^+Cl^- se ha detectado en la membrana basolateral de la porción delgada del asa descendente (77). También se tiene evidencia funcional de este transportador en células endoteliales (55); estudios recientes de hibridación *in situ* con una sonda para KCC1 de reacción cruzada sugieren la presencia de KCCs en el endotelio de las arteriolas renales (78). Además de un papel en la regulación del volumen celular, el flujo localizado de K^+ a través del cotransportador de K^+Cl^- puede regular el tono vascular (79) y así la filtración glomerular. Las células mesangiales glomerulares expresan el cotransportador de $Na^+K^+2Cl^-$ (CSB2/NKCC1) (80, 81) y también parecen expresar KCCs (78). El tratamiento de células mesangiales con furosemida reduce el $[Cl^-]_i$ y atenúa la activación del canal de Cl^- por angiotensina II (82). Como en las neuronas (53, 83-85), un balance entre cotransportadores cloro-

catiónicos opuestos puede regular el Cl^- glomerular, que juega un papel central en la fisiología glomerular (86, 87).

Biología molecular de la familia de los cotransportadores electroneutros acoplados a cloro (CCCs)

El cotransportador de $\text{Na}^+:\text{Cl}^-$ sensible a tiazidas fue el primer cotransportador electroneutro que se identificó a nivel molecular. La clonación se llevó a cabo a partir de la vejiga urinaria de un teleosteo, el lenguado de invierno *Pseudopleuronectes americanus*. Embriológicamente la vejiga urinaria es una extensión del riñón, donde se reabsorbe NaCl a través de un mecanismo de transporte electroneutro interdependiente de Na^+ y Cl^- que no requiere de la presencia de K^+ y es sensible a diuréticos del tipo de las tiazidas (88, 89). Molecularmente el DNAC que codifica para el cotransportador de $\text{Na}^+:\text{Cl}^-$ sensible a tiazidas de 3.7 kb fue identificado de la vejiga urinaria del lenguado mediante la estrategia de clonación por expresión funcional en los ovocitos de la rana *Xenopus laevis* y se le denominó CSTfl (cotransportador sensible a tiazidas del flounder) (90). A partir de entonces, como muestra el árbol filogenético de la figura 9, en los últimos años se han identificado molecularmente ocho genes que codifican para cuatro grupos de cotransportadores electroneutros acoplados a cloro (CCC). Esta clasificación está basada en el catión que es acoplado al cloro para su translocación, la estequiometría del transporte y la sensibilidad a inhibidores: El cotransportador de $\text{Na}^+:\text{Cl}^-$ sensible a tiazidas (CST/NCC); los cotransportadores de $\text{Na}^+:\text{K}^+:\text{2Cl}^-$ y de $\text{Na}^+:\text{Cl}^-$ sensibles a bumetanida (CSB/NKCC); un transportador denominado CCC8 o CIP1 (proteína de interacción con CCC) y finalmente, los

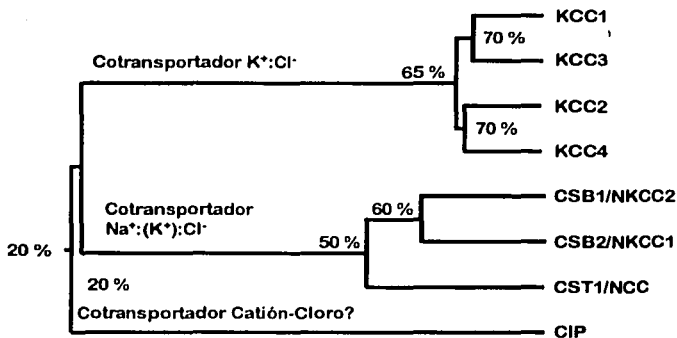


Figura 9. Árbol filogenético de la familia de los cotransportadores electroneutros

cotransportadores de K^+Cl^- sensibles a furosemida y DIOA (KCC) (91). Con respecto a CIP1, este muestra ~ 25% de identidad en la secuencia de aminoácidos con cada uno de los otros cotransportadores cloro-catiónicos conocidos. CIP1 codifica para una proteína de 914 residuos de aminoácidos y 96.2 kDa y está presente en músculo, placenta, cerebro y riñón. Caron y colaboradores sugirieron que CIP1 forma parte de una nueva familia de proteínas que modifican la actividad o cinética de los CCC a través de la formación de heterodímeros. Sin embargo, hasta la fecha no se ha determinado el catión al cual está acoplado el cloro (92).

El análisis de la secuencia primaria de los CCC indica que son proteínas de aproximadamente 770-1200 residuos de aminoácidos que comparten una estructura secundaria similar. El análisis de hidrofobicidad, según el algoritmo propuesto por Kyte y Doolittle que se muestra en la figura 10 (93), predice la existencia de un dominio central hidrofóbico, con 12 regiones alfa-hélices correspondientes a segmentos transmembranales (STM), flanqueado de dos

dominios, amino y carboxi terminales, predominantemente hidrofílicos, con múltiples sitios potenciales para fosforilación vía proteinaas cinasas A y C, y de localización posiblemente intracelular. La homología es mayor en los STM, aunque algunas de las asas intracelulares y gran parte del carboxi terminal

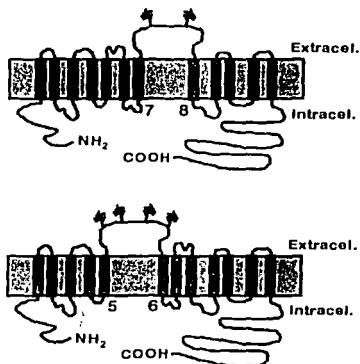


Figura 10. Topología básica de los cotransportadores electroneutros

también están altamente conservados. Recientemente,

Gerelsaikhan y Turner demostraron que la topología propuesta con 12 regiones transmembranales es la correcta para el CSB2/NKCC1 (94).

Existe una gran asa hidrofílica entre los STM 7 y 8 en aquellos cotransportadores que unen Na^+ y entre los STM 5 y 6 en CIP y en los

KCCs. Ésta estructura tiene dos sitios potenciales de glucosilación

conservados entre todos los CCC, y recientemente se demostró que el cotransportador de $\text{Na}^+:\text{Cl}^-$ de la rata (CSTr) está glucosilado, lo cual sugiere que el asa es una estructura claramente extracelular (95). La variación en el número de sitios de N-glucosilación en el asa extracelular de los KCCs determina que la masa molecular varíe entre 150 y 160 kDa y 110 y 120 kDa para las proteínas no glucosiladas, respectivamente (1).

Los sitios de unión o transporte de K^+ se sospecha que se localiza en el STM2, mientras que los STM4 y STM7 pueden ser responsables de la unión a

aniones debido a que contienen muchos residuos conservados entre los KCCs, CSB/NKCC y CST/NCC.

Características moleculares de la subfamilia de los cotransportadores electroneutros de potasio-cloro

A partir de las bases de datos genómicas, se han identificado y clonado, considerando su semejanza con otros cotransportadores electroneutros cloro-catiónicos, cuatro genes que codifican para las isoformas del cotransportador de K^+Cl^- , denominados KCC1, KCC2, KCC3 y KCC4.

Los DNAC obtenidos para KCC1 se han clonado a partir de riñón de conejo, humano y cerebro de rata (96), riñón de cerdo (97) y de eritrocitos de ratón (98, 99), con estructuras génicas reportadas para humano (97) y ratón (100). KCC2 ha sido clonado de cerebro de rata (101) y recientemente de cerebro humano (datos no publicados). Dos isoformas generadas por empalme alternativo de exones para KCC3 humano clonado de células vasculares endoteliales (102), placenta (103) y cerebro humano (104) y KCC4 identificado de riñón en humano y ratón (104).

Los cotransportadores de K^+Cl^- no son exclusivos de mamíferos, también se han identificado en otras células como en los eritrocitos y las células hepáticas de teleosteos (105-108) así como en los glóbulos rojos de anfibios (109) y recientemente nosotros identificamos en los ovocitos de *Xenopus* una secuencia parcial de DNAC que codifica para una isoforma de KCC perteneciente a la subfamilia KCC1/3 (110). En invertebrados, por ejemplo, se tiene evidencia de los KCCs en las neuronas de langosta (111), túbulos de

malpiglio de *Drosophila melanogaster* (112), en la hormiga de bosque *Formica polyctena* (113) y en el nemátodo *Caenorhabditis elegans* (97).

KCC1

En 1996 Gillen y colaboradores (96) reportaron la clonación molecular del primer cotransportador de K^+Cl^- , denominado KCC1. Ésta identificación se realizó mediante la búsqueda de secuencias homólogas con el cotransportador basolateral de $Na^+K^+2Cl^-$ (CSB2/NKCC1) en la base de datos de los *expressed sequence tags* de humano (hESTs). KCC1 codifica para una proteína de 1085 residuos de aminoácidos con una homología en secuencia de ~25% de identidad con los cotransportadores de $Na^+K^+2Cl^-$ sensibles a bumetanida (CSB/NKCC) y de Na^+Cl^- sensible a tiazidas (CST/NCC). Al igual que el resto de los miembros de la familia de los CCC, el análisis de hidropatía de KCC1 incluye 12 segmentos transmembrana (STM), una gran asa extracelular con sitios potenciales para *N*-glucosilación (con cuatro sitios conservados entre rata, conejo y humano) y regiones amino y carboxi terminales citoplásmicas.

El cotransportador de K^+Cl^- es regulado por eventos de fosforilación y desfosforilación. De hecho, la secuencia de KCC1 presenta algunos sitios consenso para proteínas cinasas que incluyen diez sitios para caseína cinasa II y cuatro sitios para PKC. KCC1 es una isoforma de amplia distribución que expresa un transcrito de 3.8 kb. Este patrón de expresión ubicuo es consistente con la vasta presencia de ESTs de KCC1 en la base de datos y sugiere que KCC1 representa una isoforma "housekeeping" responsable de la regulación del volumen celular y de la reabsorción transepitelial de K^+ y Cl^- en el riñón humano (78). Cuando se expresa en células de riñón de embrión humano (HEK

293), KCC1 produce una glucoproteína de ~ 150 kDa. Los ensayos de eflujo con $^{86}\text{Rb}^+$ en células HEK 293 transfectadas con KCC1 muestran que dicho transportador es estimulado por aumento del volumen celular (7 veces más que el eflujo de las células control) e inhibido por 2 mM de furosemide. Cuando los ensayos de captación de $^{86}\text{Rb}^+$ se llevaron a cabo en isotonicidad, previo tratamiento con 1mM de NEM, KCC1 mostró una activación significativa que fue dependiente de la presencia de Cl^- pero no de Na^+ externo y fue inhibida por la adición de furosemida y bumetanida. En el humano, KCC1 se localiza en el cromosoma 16q22.1.

KCC2

La segunda isoforma del cotransportador de $\text{K}^+:\text{Cl}^-$, KCC2, fue identificada también mediante el análisis de bibliotecas de DNAc y búsquedas en bases de datos (101). KCC2 fue clonada a partir de cerebro de rata y su distribución está restringida al sistema nervioso central (SNC). KCC2 codifica para una proteína de 1116 residuos de aminoácidos con una masa molecular de 123.6 kDa. La secuencia de aminoácidos deducida para KCC2 es 67% idéntica a la de KCC1 y sólo 25% a CSB/NKCC y CST/NCC. Como todos los miembros de la familia de los CCC, comparte la misma estructura secundaria. El análisis de northern blot revela un transcrito de ~5.6 kb exclusivamente en el cerebro y la hibridación *in situ* muestra que KCC2 se expresa particularmente en neuronas, incluyendo las neuronas piramidales del hipocampo, las células granulares y de Purkinje en el cerebelo, entre otras. KCC2, constituye una isoforma específica del cerebro y su alta afinidad por el catión ($K_m \text{ K}^+ 5.2 \text{ mM}$) sugiere que puede funcionar como una vía de influjo y eflujo, manteniendo una baja concentración de cloro intraneuronal que repercute en el efecto inhibitor/excitador del

neurotransmisor GABA. Cuando se expresa en células HEK 293 o en ovocitos de *Xenopus*, ésta isoforma expresa actividad constitutiva en isotonicidad, la cual aumenta durante la hipotonicidad (114, 115). En ambos casos la captación de $^{86}\text{Rb}^+$ es dependiente de Cl^- pero no de Na^+ externo y sensible a diuréticos de asa. KCC2 se localiza en el cromosoma humano 20 (116).

El DNAC que codifica para la isoforma de KCC2 en el humano (hKCC2) fue clonada recientemente por nuestro grupo. El DNAC de hKCC2 mide 5.9 kb con un marco de lectura abierto de 3348 nucleótidos y tiene una identidad del 90 y 99% en secuencias de nucleótidos y residuos de aminoácidos, respectivamente con la secuencia de la rata. La expresión del RNAC de hKCC2 en ovocitos de *Xenopus laevis* resulta en la captación de $^{86}\text{Rb}^+$ dependiente de Cl^- en isotonicidad; el aumento de volumen celular en hipotonicidad causa su activación en 20 veces y es bloqueada por caliculina A, un inhibidor de proteínas fosfatasa.

La cinética del transporte de iones a través de hKCC2 en condiciones isotónicas e hipotónicas, mostró K_m s para K^+ y Cl^- de 9.3 ± 1.8 mM y 6.8 ± 0.9 mM, respectivamente; ambas afinidades son significativamente mayores que para KCC1. La localización genómica de hKCC2 se determinó en el cromosoma 20q13, entre los marcadores D20S836 y D20S888 (datos no publicados).

KCC3

KCC3 muestra al menos dos isoformas por mecanismo de empalme alternativo, generadas por dos exones separados que codifican para el primer exón y difieren en el contenido y distribución de sitios potenciales para fosforilación (104). Éstas isoformas, denominadas KCC3a y KCC3b muestran

transcritos en corazón, cerebro, músculo esquelético, riñón, placenta, pulmón, hígado y páncreas. Dada su distribución tisular, KCC3 probablemente cumpla varias funciones: puede estar involucrado como una vía de influjo en la regulación del volumen celular y el transporte epitelial de sal y agua, mientras que en los tejidos donde se expresa ampliamente como corazón, músculo esquelético y cerebro, puede funcionar como regulador o amortiguador de las concentraciones de K^+ y Cl^- en las células y el espacio intersticial (103).

KCC3b (102) fue identificado mediante PCR de cambio diferencial (PCR-DD), una metodología comúnmente usada para aislar genes regulados en células endoteliales por factores de crecimiento como el factor angiogénico del endotelio vascular (FAEV). El análisis del RNA total extraído de cordones umbilicales humanos tratados con FAEV, mediante PCR-DD, mostró una de las bandas consistentemente regulada por FAEV. Esto permitió la identificación de un DNAc que codifica para una proteína de 1099 residuos de aminoácidos y es 77% idéntico a KCC1 humano y 73 % a KCC2. El perfil de hidropatía mostró el mismo patrón que para el resto de los KCCs: 12 STM, una gran asa extracelular con cinco sitios potenciales para *N*-glucosilación entre los STM 5 y 6, así como extremos NH_2 y $COOH$ terminales. KCC3b carece de un residuo de ácido glutámico al principio de la secuencia del STM 2. Se ha propuesto que este residuo sea importante para aumentar la unión del potasio externo en KCC2 (114). El mRNA de KCC3b se expresa en cerebro, corazón, músculo esquelético y riñón, con transcritos de 9, 7.5 y 4.5 kb. La expresión del DNAc de KCC3b en células HEK 293 produce una glucoproteína de ~150 kDa que es reducida por digestión con glucosidasa hasta 120 kDa. La captación de $^{86}Rb^+$ en las células transfectadas con KCC3b mostró dependencia de Cl^- pero no de

Na⁺ externo y los diuréticos de asa inhibieron de manera dependiente de la dosis, siendo ligeramente mayor con furosemida (K_i ~10 μM) que con bumetanida (K_i ~40 μ). Además, el tratamiento con hipotonicidad no aumentó significativamente la captación de ⁸⁶Rb⁺ en aquellas células transfectadas con KCC3b. La localización cromosómica de KCC3b se determinó mediante hibridación *in situ* fluorescente en el cromosoma 15q13 y entre los marcadores genéticos D15S1010 y D15S1040. El área entre dichos marcadores se ha asociado con epilepsia mioclónica juvenil (117) por lo que KCC3b es un gen candidato para ésta enfermedad.

KCC3a identificado en el humano se localiza en el cromosoma 15q14 entre los marcadores D15S1040 y D15S118. Ésta sección del cromosoma 15 está ligada a dos subtipos de epilepsia idiopática generalizada (117, 118) y a un fenotipo neurofisiológico asociado con esquizofrenia (119). Además están cerca de una región que contiene el gen de la neuropatía periférica con o sin agénesis del cuerpo calloso, conocido como síndrome de Andermann (120). KCC3a es la isoforma más grande de todos los KCCs debido a que presenta una porción de ~60 residuos de aminoácidos en el extremo amino terminal. La secuencia de KCC3 predice un total de dos sitios potenciales para forforilación por PKA y 11 sitios para PKC, siete de ellos localizados en los primeros 90 residuos de aminoácidos (104).

KCC4

Por último KCC4 (104), localizado en el cromosoma 5p15.3, entre los marcadores D5S678 y el telómero, se distribuye ampliamente en corazón y riñón; en éste último, puede estar relacionado con el transporte iónico basolateral en la porción gruesa del asa ascendente de Henle (57, 121) y con

aquellos casos de síndrome de Bartter no asociados a mutaciones en CSB1/NKCC2, ROMK y CLC-NKB (122) (Ver tabla 3).

Tabla 3. Características moleculares de los cotransportadores de K⁺:Cl⁻

CARACTERÍSTICAS	K ⁺ :Cl ⁻	K ⁺ :Cl ⁻	K ⁺ :Cl ⁻	K ⁺ :Cl ⁻	K ⁺ :Cl ⁻
Nombre	KCC1	KCC2	KCC3a	KCC3b	KCC4
Fuente de clonación	rata conejo humano cerdo ratón	rata humano	ratón humano	ratón humano	ratón humano
Proteína (aa)	1086	1116, 1095	1150	1099	1083
Peso (kDa)	150	123.6	150	150	115
Empalme alternativo	--	--	Con exón 1	Sin exón 1	--
Distribución tisular	Ubicua	SNC	Varios	Varios	Varios
Transcrito (kb)	3.8	5.6	6, 7	9, 7.5, 4.5	5.3
Número de exones	24	24	26	25	24
Localización cromosómica	16q22.1	20q13	15q14	15q14	5p15.3
Genes candidatos de enfermedades humanas	Anemia Falciforme	Epilepsia	Síndrome de Andermann, Catatonia	Síndrome de Andermann, Catatonia	Síndrome de Bartter

Hipótesis

Dado que existe una gran homología con KCC1 y KCC2, para los cuales la expresión funcional ha sido reportada (96, 101), se espera que KCC3 y KCC4 codifiquen para cotransportadores de K^+Cl^- .

Objetivo

Establecer las propiedades funcionales de KCC3 y KCC4, así como extender y definir las propiedades funcionales de cada isoforma en mayor detalle, utilizando el sistema de expresión heteróloga de las isoformas individuales en ovocitos de la rana *Xenopus laevis*. Se espera que la comparación de los cuatro KCCs proporcione importantes pistas acerca de la relación entre la estructura y la función de estos y otros cotransportadores cloro-catiónicos.

METODOLOGIA

Identificación molecular de KCC3 y KCC4

Usando la base de datos de los *expressed sequences tags* (EST) de humano y ratón como punto de partida, clonamos los DNAC correspondientes a dos nuevas isoformas del cotransportador de K⁺:Cl⁻ denominadas hKCC3 y mKCC4, respectivamente.

Clonación del DNAC

Una búsqueda en la base de datos de los EST reveló numerosos ESTs de ratón que contenían marcos parciales de lectura homólogos a las secuencias reportadas previamente para KCC1 y KCC2 de rata. Los DNAC correspondientes a cuatro de estos ESTs [568084, 633794, 313521, 698105] se obtuvieron del consorcio IMAGE y se secuenciaron. El extremo 3' incluyendo la región no codificable se identificó a partir de un quinto DNAC (clona 1314687). La distribución tisular de mKCC4 se probó por RT-PCR y su expresión se detectó con los primers S1/AS1, con un transcrito particularmente abundante en riñón y corazón. Usando los primers S2/AS2, el hueco entre las clonas 313521 y 568084, se amplificó un fragmento de 1.2 kb a partir de riñón de ratones C57BL6J que se subclonó en el sitio *Eco* RV de pBluescript por ligación de extremos romos. Las condiciones para la PCR de estos y otros primers específicos para cada gen se optimizaron usando *Taq* 2000 y el sistema de buffers Opti-Prime (Stratagene). El protocolo de amplificación fue el siguiente: 30 ciclos de desnaturalización (92 °C, 2 min), alineación (54 °C, 1 min) y extensión (72 °C, 1 min), seguido por un paso de extensión final (72 °C, 8 min). El extremo 5' de mKCC4 se clonó a partir de riñón de ratones BALB/c como templado para el 5'-RACE (rapid amplification of conserved ends)

CLONTECH, usando dos primers antisentido AS3/AS4 y el primer adaptador AP1 de CLONTECH (primer S3). Ésta PCR utilizó AmpliTaq-Gold DNA polimerasa (Perkin-Elmer) y un protocolo de amplificación de inicio caliente, consistente en un paso de activación de la enzima por 9 min a 94 °C, seguido por 35 ciclos de 94 °C por 1 min y 68 °C por 2.5 min y 10 min finales de extensión a 72 °C.

La secuencia de la clona 150738 EST de KCC humano reveló que este DNAC es ortólogo de mKCC4. Otro EST 5' humano (Genbank F12342) mostró homología significativa en aminoácidos con el extremo 3' de la secuencia codificada por otros KCCs. Este EST se traslapa con un gran número de ESTs del extremo 3' no codificable de hKCC4, incluyendo las clonas IMAGE 22250 y 51311. El hueco entre estos DNAC y la clona 150738 fue rellenada con RT-PCR con los primers S4/AS5, usando riñón humano como templado (CLONTECH). Los productos de PCR de ésta reacción se subclonaron en pBluescript. Finalmente, el extremo 5' de hKCC4 se clonó por RT-PCR secuencial de riñón humano con los primers S5/AS6 y S6/AS7. S5 y S6 fueron derivados de las clonas IMAGE 1403108 y mKCC4, respectivamente.

El análisis de la secuencia de otro DNAC de EST humano (clona 150620) indicó la existencia de un cuarto KCC, hKCC3 y el análisis de northern blot reveló su expresión en músculo y sólo un DNAC 5'-RACE se clonó de un templado de músculo humano (CLONTECH) usando los primers S3/AS8. Para ésta PCR 5'-RACE se utilizó la Taq DNA polimerasa Advantage (CLONTECH) y un protocolo de inicio caliente como sigue: 94 °C por 1 min, seguido de 35 ciclos de 94 °C por 30 seg, 62 °C por 30 seg y 68 °C por 3 min. El análisis de una biblioteca de DNAC de músculo humano (CLONTECH) con una sonda

comprendida entre los nucleótidos 741-871 de hKCC3 produjo sólo un DNac que se extendió 5' del codón de inicio. Finalmente, dos fragmentos de PCR traslapados se amplificaron a partir de cerebro humano (CLONTECH) con los primers S7/AS9 y S8/AS10 y se subclonaron en pCR2.1 por TA clonning (Invitrogen).

Tabla 4. Oligonucleótidos usados como primers en la PCR

Nombre	Secuencia	Sitio inicio	Clona
S1	5'- GGAGGCAGATAATCCTTTCTCC - 3'	2472	mKCC4
S2	5'- GATCTTCTGACCTACATCTCTCC - 3'	738	mKCC4
S3	5'- CCATCCTAATACGACTCACTATAGGGC - 3'		AP1
S4	5'- GACTCGTTTCCGCAAACC - 3'	2498	hKCC4
S5	5'- GGAAGTAAACATGGCACTTTTCG - 3'	200	hKCC4
S6	5'- AGCCATGCCACGAACTTTACGG - 3'	69	mKCC4
S7	5'- AGTCAGAACTCCATCACAGGG - 3'	441	hKCC3
S8	5'- CTATCCTTGCCATCCTGACC - 3'	1723	hKCC3
S9	5'- CACACAGTTCAGCCTCTGACC - 3'	4241	hKCC3
S10	5'- CTGAATGACCGTGCTTGACC - 3'	3624	hKCC3
S11	5'- CACGCTGAGCAGTCAAAGGC - 3'	3858	hKCC3
AS1	5'- CACAGCAGTAGCATCCTCC - 3'	3135	mKCC4
AS2	5'- ACACTGCTCCGAGTCCAGG - 3'	2187	mKCC4
AS3	5'- ACCGCCATGAGTGCCAGGGCACAGCTGC - 3'	863	mKCC4
AS4	5'- TTCCTAGAAGGCAGACCGGGATGTCAGGTG - 3'	1007	mKCC4
AS5	5'- AGAGTGCCGTGATGCTGTTGG - 3'	3280	hKCC4
AS6	5'- CAGGGCCATGAGCACGAGCGTG - 3'	796	hKCC4
AS7	5'- GAAATGGCGGTCACATTGTAC - 3'	513	hKCC4
AS8	5'- CCTCAGAAAATTGCTCCACATTGCTGG - 3'	2889	hKCC3
AS9	5'- GAAGGAGCCAATAACAATCACC - 3'	1895	hKCC3
AS10	5'- GCAGCAGTTGTCACTCGAAC - 3'	2821	hKCC3
AS11	5'- GCAACCTCTTTATGGACAACC - 3'	4618	hKCC4
AS12	5'- GCAGCATGTTCAAGTTAGTATCC - 3'	4185	hKCC3

Análisis de la secuencia de los DNac de KCC3 y KCC4

Todas las clonas de DNac se secuenciaron en ambos sentidos usando el colorante fluorescente de terminación química (Applied Biosystems) con el siguiente protocolo: 25 ciclos de 95 °C por 30 seg, 50 °C por 15 seg y 60 °C por 4 min. Para las secuencias de DNac derivadas exclusivamente de PCR, al

menos dos DNAs de dos reacciones separadas de PCR se secuenciaron. El análisis de las secuencias de nucleótidos y aminoácidos se realizaron usando el programa GeneWorks 2.5 y MacVector 6.5 (Oxford Molecular). Las alineaciones y otros análisis también se hicieron usando los programas computacionales BLAST, DNASTar y SMART (simple modular architecture research tool).

Localización cromosómica y estructura genómica de las isoformas KCC3 y KCC4 en el humano

La localización cromosómica de los genes KCC3 y KCC4 humanos se realizó mediante un análisis de PCR con el *National Institute of General Medical Sciences human/rodent somatic cell hybrid*. Los primers usados para mapear hKCC4 (S9 y AS11) amplificaron un segmento de 377 pb de la región 3' no codificante del DNA genómico y el hKCC3, mapeado con los primers S10 y AS12 amplificando un segmento de 561 pb de la secuencia 3' no codificante. Las reacciones de PCR usando DNA se corroboraron por la presencia o ausencia de los productos de tamaño apropiados usando electroforesis en geles de agarosa.

La localización cromosómica de hKCC4 se verificó por secuenciación del cromosoma 5 clona pMS621. El mapeo de hKCC3 se realizó por PCR usando análisis de radiación de híbridos con el panel Stanford G3 y los primers S10/AS12. Los productos de la reacción generados por PCR se desnaturalizaron y se aplicaron a membranas de nylon para análisis de Southern blot usando como sonda un oligonucleótido interno marcado con ^{32}P (primer S11).

Extracción de RNA total

El RNA total se extrajo mediante la técnica de homogeneización con isotiocianato de guanidina y ultracentrifugación en cloruro de cesio. El RNA extraído se cuantificó por espectrofotometría de luz ultravioleta a una absorbancia de 260nm y 280nm en un programa de ácidos nucleicos (DU640 spectrophotometer Beckman) y su calidad se determinó por la aparición de las subunidades 28S y 18S del RNA ribosomal en electroforesis de agarosa-formaldehído al 1% teñido con bromuro de etidio. El RNA total se precipitó con 0.1v de acetato de sodio pH 5.2 y 3v de etanol al 100 % y se guardó a -20 °C.

Análisis de northern-blot para hKCC3a y mKCC4

El análisis de la expresión génica de las isoformas hKCC3a y mKCC4 del cotransportador de K⁺:Cl⁻ se realizó utilizando de membranas cargadas con RNA poli(A)⁺ de diversos tejidos humano (CLONTECH) o con el RNA total extraído de cada uno de los tejidos obtenidos a partir de ratones C57BL6J, respectivamente. El análisis se llevó a cabo con 2 µg de poli(A)⁺ o 10 µg de RNA total por carril en condiciones de baja estringencia. Para realizar este análisis primero se corrió un gel de agarosa/formaldehído con 10 µg de RNA total de cada tejido de ratón, se transfirió a una membrana de nylon por capilaridad y posteriormente el RNA transferido se fijó a la membrana con luz ultravioleta en un UV Stratalinker 2400.

El análisis se realizó utilizando como sondas la fracción 4417-5062 del extremo 3' no codificable de mKCC4 y GA3PD como control, ambas marcadas con ³²P. El northern blot para los tejidos humanos que contenían 2 µg/carril de RNA poli(A)⁺ (CLONTECH) se hibridó con las sondas generadas por PCR de los extremos 3' no codificables de hKCC4 (nucleótidos 4598-4957) y hKCC3

(nucleótidos 3624-4185) y con β -actina humana como control. La membrana se expuso en una placa radiológica a temperatura ambiente y el tiempo de exposición varió dependiendo de la sonda y del tejido utilizados. Las bandas resultantes se midieron mediante análisis densitométrico de las imágenes digitalizadas de las placas radiológicas.

Transcripción *in vitro* de RNAc

El DNAc que codifica para cada clona se insertó dentro del vector de alta expresión pGEMHE. Este vector se diseñó para expresar copias de RNAc a un alto nivel en ovocitos de *Xenopus laevis*, con un sitio de clonación múltiple flanqueado por la β -globina de *Xenopus* en el extremo 5' y en el 3' no codificable. Para sintetizar el RNAc, cada clona se linearizó en el extremo 3' (KCC1-2-4 con *Nhe* I y KCC3a con *Not* I) y el RNAc se transcribió *in vitro* usando el promotor T7 de la RNA polimerasa usando el kit mMMESSAGE (Ambion). La calidad del RNAc se corroboró en electroforesis de geles de agarosa-formaldehído.

Análisis de la expresión funcional de cotransportadores electroneutros

El ensayo básico empleado para medir la expresión funcional de proteínas de membrana es la captación de isótopos radioactivos por cada ovocito. El material radioactivo se utiliza debido a que los cotransportadores de K^+Cl^- son electroneutros, por lo que no puede medirse la expresión mediante el registro de cambios en el potencial transmembranal. Para medir el transporte de K^+Cl^- los radioisótopos que pueden usarse son el $^{86}Rb^+$ y el $^{36}Cl^-$. El $^{86}Rb^+$ atraviesa las membranas celulares por las mismas vías de transporte que el K^+ . De estos isótopos empleamos el $^{86}Rb^+$, ya que su vida media es de 18 días, mientras que la del $^{36}Cl^-$ es de 300,000 años.

Obtención, preparación e inyección de los ovocitos de *Xenopus laevis*

Ranas hembra *Xenopus laevis* se anestesiaron por inmersión en tricaina 0.17% durante 30 min, después de los cuales se extraen varios lóbulos del ovario mediante una pequeña incisión en el abdomen. Posteriormente los ovarios se incuban en solución Ringer de rana sin Ca^{2+} a la cual se le añade colagenasa (Roche tipo B, 2 mg/ml) durante 60 min. Al término de este tiempo se separan los ovocitos maduros (estadios V-VI) y la capa folicular se remueve manualmente con pinzas finas.

Una vez desfolliculados, los ovocitos se incuban durante una noche en Ringer de rana (ND96 en mM: NaCl 96, KCl 2, CaCl_2 1.8, MgCl_2 1, HEPES/Tris 5, pH 7.4), suplementado con 2.5 mM de piruvato de sodio y 5 mg/100 ml de gentamicina, a 17 °C. Al día siguiente se inyectan con agua (50 nl/ovocito) o con RNAc (25 ng/ovocito en 50 nl de Tris 1mM, pH 6.2). Después de inyectados, los ovocitos se incuban de 3 a 4 días a 17 °C en ND96 para dar tiempo a que se produzca la proteína y se lleven a cabo las modificaciones post-traduccionales que incluyen la inserción en la membrana celular.

Ensayo de captación para medir la expresión funcional de los cotransportadores de $\text{K}^+:\text{Cl}^-$ en ovocitos

Debido a que el cotransportador de $\text{K}^+:\text{Cl}^-$ es activado por aumento de volumen celular, utilizamos soluciones isotónicas e hipotónicas para medir su expresión. La captación de $^{86}\text{Rb}^+$ se midió después de un periodo de 30 min en una solución isotónica o hipotónica libres de Na^+ y Cl^- . La solución hipotónica (~125 mosmol/kg) contenía en mM: gluconato de *N*-metil-D-glucamina 48, gluconato de K^+ 2, gluconato de Ca^{2+} 4, gluconato de Mg^{2+} 1 y HEPES/Tris 5, pH 7.4. Para la solución isotónica (~220 mosmol/kg) se añadió sacarosa a una

concentración de 100 mM. A ambas soluciones se les agregó ouabaina a una concentración de 1 mM, para inhibir el transporte generado por la bomba de Na^+/K^+ . Posteriormente, los ovocitos se transfirieron, durante 60 min, a una solución de captación libre de Na^+ isotónica o hipotónica. La solución hipotónica (~125 mosmol/kg) contenía en mM: *N*-metil-D-glucamina-Cl⁻ 48, KCl 2, CaCl₂ 1.8, gluconato de MgCl₂ 1 y HEPES/Tris 5, pH 7.4. Nuevamente a la solución isotónica (~220 mosmol/kg) se le añadió sacarosa 100 mM. Ambas soluciones de captación contenían 5 $\mu\text{Ci/ml}$ de $^{86}\text{Rb}^+$ (New England Nuclear) y 1 mM de ouabaina. Al término de este periodo, los ovocitos se lavaron cinco veces en la misma solución de captación fría (sin el material radioactivo) y en forma individual se colocaron en tubos de centelleo líquido que contenían SDS al 10% para lisar la membrana y liberar la radioactividad captada. Dicha actividad se determinó en un contador de centelleo β .

Resultados y Discusión

La necesidad de regular rápidamente el volumen celular después del encogerse o hincharse osmóticamente es de vital importancia para las células de organismos inferiores, que continuamente están expuestas a estrés osmótico. Las células de los mamíferos también están sujetas a cambios de volumen y aún cuando la osmolaridad del medio interno es finamente regulada, pueden mostrar respuestas rápidas para mantener su volumen.

Éstas respuestas están dadas por la activación de proteínas de membrana que median el transporte de iones a través de la membrana celular. Entre los mecanismos empleados por la célula durante el aumento de volumen se encuentran los cotransportadores electroneutros de K^+Cl^- , que permiten la salida de iones acompañada de agua y por lo tanto la recuperación del volumen celular.

Los eritrocitos fueron las células en las que originalmente se determinó la presencia de un sistema de transporte de K^+ dependiente de Cl^- e independiente de Na^+ . Este transporte de iones es interdependiente, es decir, el movimiento de los iones está acoplado y por lo tanto es electroneutro e independiente del potencial transmembranal y en muchas células el KCC funciona como una vía de eflujo de K^+ . En los eritrocitos este cotransportador puede estimularse por aumento del volumen celular, la activación de proteínas cinasas, la inhibición de proteínas fosfatasa y el agente N-etilmaleimida. La disminución del volumen celular y los derivados de diuréticos de asa como la furosemida y de los estilbenos como el DIDS actúan como inhibidores del cotransportador de K^+Cl^- (7, 25).

Debido a la gran heterogeneidad molecular y fisiológica del cotransporte de K^+Cl^- ha sido difícil determinar sus propiedades funcionales, farmacológicas y fisiológicas. Sin embargo, este panorama ha cambiado recientemente con la identificación de los DNAc que codifican para dos cotransportadores de K^+Cl^- , KCC1 y KCC2. Las proteínas codificadas por ambas secuencias de DNAc son homólogas a los otros cotransportadores cloro-catiónicos electroneutros, los cotransportadores de $Na^+:K^+:2Cl^-$ sensibles a la bumetanida (CSB/NKCC) y el cotransportador de $Na^+:Cl^-$ sensible a las tiazidas (CST/NCC) (91).

En éste trabajo se muestra la clonación del DNAc que codifica para dos isoformas del cotransporte de K^+Cl^- : KCC3a del humano (hKCC3a) y KCC4 del ratón (mKCC4) (104), ambos nuevos miembros de la familia génica de los cotransportadores electroneutros cloro-catiónicos, y es el primer trabajo que reúne una serie de datos detallados sobre las características funcionales de ésta subfamilia, así como la comparación directa entre las dos subramas que conforman a los cotransportadores de K^+Cl^- .

Para poder definir las principales propiedades funcionales, farmacológicas y reguladoras de las diferentes isoformas del cotransporte de K^+Cl^- utilizamos el conocido sistema de expresión funcional en ovocitos de *Xenopus laevis*. Los ovocitos de las ranas sudafricanas *Xenopus* se han utilizado ampliamente para la clonación de proteínas de membrana debido a que pueden microinyectarse con el RNAc sintetizado *in vitro*, a partir del DNAc que codifica para la proteína en estudio. Cerca del 95% de los ovocitos utilizados expresan adecuadamente la información genética que porta el RNAc microinyectado. Otra ventaja de este sistema de expresión heteróloga es que es posible modificar su medio ambiente cambiando la solución de incubación y

por lo tanto, someterlo a diversas condiciones experimentales que simulen situaciones fisiológicas. Por último, el hecho de que se empleen en estadios maduros (V y VI), cuando las células miden ~1200 μm de diámetro, hace posible microinyectarlos y manejarlos individualmente. Más aún, en ésta especie de ranas la producción de ovocitos es continua y asincrónica, de tal manera que es factible encontrarlos en diferentes estadios de madurez (I-VI) (90).

En el presente estudio las soluciones externas a las que fueron expuestos los ovocitos después de la microinyección del RNAC de cada isoforma de los KCCs, fueron soluciones isotónicas o hipotónicas (~220 y 110 mOsm/kg, respectivamente), ambas en ausencia de Na^+ externo y en presencia de ouabaína. La ausencia de Na^+ externo impidió la captación de $^{86}\text{Rb}^+$ o el intercambio de $^{86}\text{Rb}^+/\text{K}^+$ a través del cotransportador de $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ (CSB2/NKCC1), que existe en los ovocitos, ya que este cotransportador requiere de la presencia simultánea de los tres iones para funcionar y además es inhibido cuando es expuesto a soluciones hipotónicas. Por otro lado, la ouabaína previene la captación de $^{86}\text{Rb}^+$ a través de la bomba de Na^+/K^+ (123).

En medio isotónico sólo el KCC2 mostró una captación de $^{86}\text{Rb}^+$ significativa, dependiente de Cl^- externo, como se muestra en el recuadro de la figura 11, lo que significa que ésta isoforma tiene una función basal como ya había sido reportado por Payne (114). En la misma figura 11, la expresión heteróloga del DNAC de cada isoforma en medio hipotónico indujo, con diferente magnitud, la expresión de una vía de captación de $^{86}\text{Rb}^+$ activada por hinchamiento celular y dependiente de la presencia de Cl^- externo (KCCs: 4>3>1>2). Estos hallazgos difieren de observaciones previas (96, 97, 102, 103,

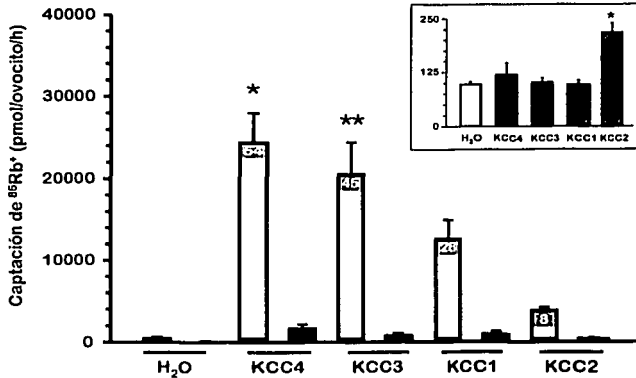


Figura 11. Efecto de la tonicidad del medio externo sobre la captación de $^{86}\text{Rb}^+$ en ovocito de *Xenopus* Inyectados con agua o con RNAc de las isoformas KCC1, 2, 3a y 4, clonada respectivamente a partir de conejo, humano, humano y ratón. Las captaciones se realizaron en hipotonicidad (120 mosm/kg) en presencia (barras blancas) o ausencia (barras negras) de Cl^- externo. El panel superior muestra la captación en isotonicidad (220 mOsm/kg) * $p < 0.01$ vs grupo control, ** $p < 0.001$ vs KCC4.

114) que indican una mínima activación por hipotonicidad de KCC1, KCC2 y KCC3 cuando son expresados en células HEK 293, pero concuerda con la activación inducida por aumento del volumen celular de KCC1 encontrada por Su y colaboradores (98) usando ovocitos de *Xenopus* como sistema de expresión. Por lo tanto, cuando son expresados en ovocitos de *Xenopus*, las diferentes isoformas de KCCs pueden activarse por un aumento del volumen celular. Esto sugiere que las células HEK 293 probablemente no poseen las vías de señalización apropiadas para la activación de los cotransportadores por aumento del volumen celular. En comparación, las vías de transporte reguladas por volumen recapitulan su fisiología *in vivo* cuando son expresados en ovocitos de *Xenopus*. De este modo, las vías de transporte activadas por

encogimiento celular tales como el cotransportador de $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ (123) o el canal de sodio epitelial ENaC (124) y las vías activadas por hinchamiento como el canal de K^+ activado por calcio mlK1 (125) probablemente son regulados apropiadamente en este sistema de expresión heteróloga. De los cuatro KCC, KCC4 parece ser la isoforma que exhibe la mayor activación por hipotonicidad. La captación de $^{86}\text{Rb}^+$ mediada por KCC4 en este estudio fue consistentemente mayor que el resto de los KCCs, aún cuando la cantidad de RNAc inyectado fue equivalente. Una explicación para la mayor activación por hipotonicidad en los ovocitos microinyectados con KCC4 es que el RNAc de ésta isoforma sea intrínsecamente más estable o se traduzca mejor que los otros KCC en los ovocitos. Sin embargo, se ha reportado que el KCC2 de la rata codifica para un cotransportador de $\text{K}^+:\text{Cl}^-$ con actividad significativa en soluciones isotónicas cuando se expresa en ovocitos de *Xenopus* y sólo muestra una mínima activación por aumento del volumen celular (115). Es probable por lo tanto, que muchas de las diferencias observadas en sensibilidad de volumen se deban a la variación en la estructura de las cuatro proteínas de KCC.

En el análisis cinético de la figura 12 se observa la captación de $^{86}\text{Rb}^+$ en ovocitos hinchados y microinyectados con el RNAc de cada isoforma de KCC. Este estudio muestra una vez más que tanto el K^+ como el Cl^- se requieren para la función del transportador. Los coeficientes de Hill para ambos iones fueron cercanos a la unidad en cada isoforma, lo que sugiere un proceso de transporte con una estequiometría de 1:1 y por consiguiente, un sistema de transporte electroneutro. La afinidad para los iones externos reveló una diferencia altamente significativa en cuanto a la K_m aparente para el K^+ externo. La isoforma con mayor afinidad por el K^+ es el KCC2, con una K_m de 9.3 ± 1.8

mM, que no es estadísticamente diferente de aquella reportada por Payne para la isoforma de la rata 5.0 ± 0.9 mM (114). La K_m para el K^+ fue de 17.5 ± 2.7 mM en KCC4 (126), seguida por 25.5 ± 3.8 mM para KCC1 (126) y 51.9 ± 3.8

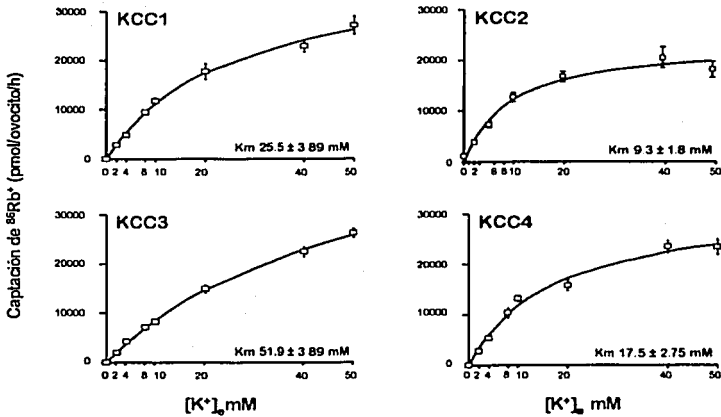


Figura 12. Análisis cinético de la activación por K^+ en ovocitos de *Xenopus* inyectados con RNAc de las distintas isoformas de KCCs.

para KCC3a (127). Aunque la dirección del transporte está determinada por los gradientes químicos de los iones transportados, se ha propuesto que los KCCs con mayor afinidad por el K^+ (KCC2 y KCC4) puedan transportar K^+ en ambas direcciones en condiciones fisiológicas o patológicas. Esto se ha verificado en neuronas, donde se expresa predominantemente KCC2 (128). En contraste, las isoformas con menor afinidad por el K^+ (KCC1 y KCC3a) funcionarían como mecanismos de expulsión, donde el gradiente de K^+ es mayor al del Cl^- en condiciones fisiológicas, de modo que favorecería el eflujo de estos iones (114).

Como se observa en la figura 13, KCC1, 3 y 4 muestran que la afinidad por Cl^- no es significativamente distinta entre sí (mM : 17.2 ± 8.3 ; 14.2 ± 3.1 ; 16.1 ± 4.2 ; $p = \text{NS}$, respectivamente), mientras que KCC2 tiene una alta afinidad por este anión ($6.8 \pm 0.9 \text{ mM}$) y es también la isoforma con mayor afinidad por la furosemida. Sin embargo, los datos cinéticos obtenidos para el KCC2 humano son muy distintos a los reportados por Payne para la misma isoforma de rata, expresada en células HEK 293 ($\text{Km K}^+ 5.0 \pm 0.9 \text{ mM}$ y $\text{Cl}^- > 100 \text{ mM}$) (114).

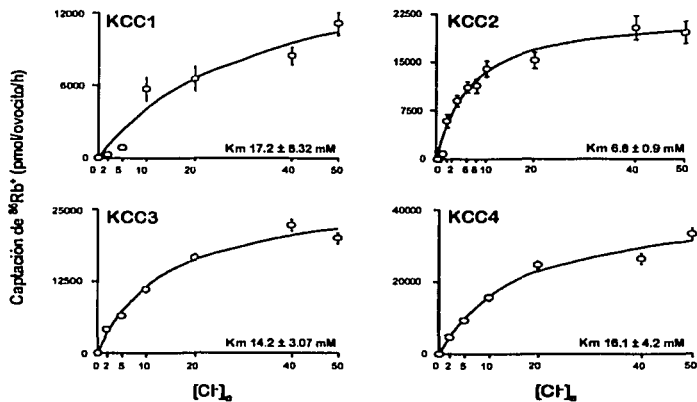


Figura 13. Análisis cinético de la activación por Cl^- en oocitos de *Xenopus* inyectados con RNAc de las distintas isoformas de KCCs.

Los KCCs difieren en la selectividad por aniones que pueden ser transportados de manera acoplada al transporte de rubidio. La figura 14 muestra la actividad relativa de los KCCs en presencia de otros aniones además del cloro (127).

En ovocitos que expresan al KCC1, la captación de $^{86}\text{Rb}^+$ es un 70% con respecto al control cuando el Cl^- es sustituido por SCN^- o Br^- externo. Para el caso del KCC2, en presencia de Br^- puede observarse cerca del 40% de la función, mientras que este mismo anión es mejor transportado que el Cl^- a

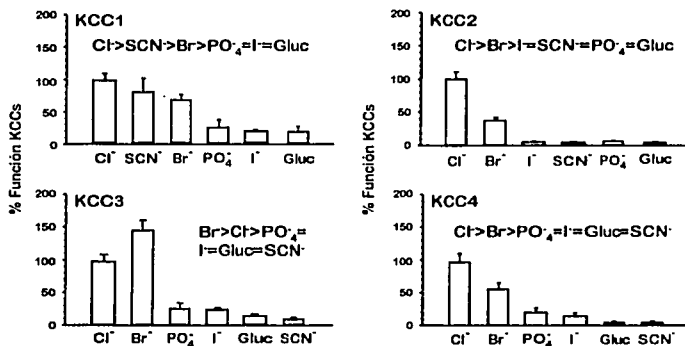


Figura 14. Expresión funcional de los cotransportadores de $\text{K}^+:\text{Cl}^-$ en presencia de 10 mM de sales de potasio para diferentes aniones sustitutos de Cl^- .

través de KCC3a. La serie presentada muestra que ninguno de los otros aniones puede ser transportado junto con el rubidio. Para KCC4 cerca del 50% de la función se obtiene en presencia de Br^- y algún transporte está aún presente con PO_4^- .

El presente estudio sugiere que las propiedades funcionales observadas para los KCCs 1, 2 y 4 expresados en ovocitos son distintas a las que caracterizan a la isoforma presente en los eritrocitos. Considerando nuestros experimentos de cinética y sustitución de aniones podemos sugerir por eliminación, que el principal cotransportador de $\text{K}^+:\text{Cl}^-$ en los eritrocitos es el KCC3 o alguna otra

isoforma aún no identificada. Lo anterior concuerda con los datos obtenidos por Delpire y Lauf (129) en los que observaron que el cotransportador de K^+Cl^- en eritrocitos de oveja LK hinchados osmóticamente tienen una K_m para K^+ externo de ~ 55 mM, muy diferente a las K_m obtenidas para KCC1, 2 y 4. Otra característica importante que se ha mostrado también en eritrocitos de oveja (130), es que el flujo de K^+ es mayor en presencia de Br^- que en presencia de Cl^- , característica que tampoco presentan KCC1, 2 y 4.

Además, aunque se ha mostrado que el RNAm de KCC1 se expresa en células eritroleucémicas de ratón, este no está presente en reticulocitos circulantes (99), y la isoforma KCC2 se expresa exclusivamente en el sistema nervioso central (101).

Los miembros de la familia de los cotransportadores cloro-catiónicos electroneutros han sido definidos en parte con base en su sensibilidad a ciertos diuréticos. Así, los cotransportadores de $Na^+K^+2Cl^-$ son sensibles a diuréticos de asa, derivados del ácido sulfamoylbenzóico y son resistentes a los derivados de las benzotiadiazinas, mientras que el transporte de Na^+Cl^- se inhibe por tiazidas pero no se afecta por los diuréticos de asa (131).

La figura 15 muestra el mejor ajuste a los datos experimentales de una curva de inhibición dosis-respuesta a la furosemida en los KCCs. Los valores de IC_{50} (μM) obtenidos para cada isoforma fueron ~ 180 , 75, 180 y 700, respectivamente para KCC1 en rojo, KCC2 en verde, KCC3a en azul y KCC4 en negro. La figura muestra que el KCC2 es el más sensible a la furosemida con un IC_{50} de ~ 70 μM , mientras que KCC4 fue el menos sensible con un IC_{50} de ~ 700 . Así pues, los cotransportadores de K^+Cl^- son sensibles a los diuréticos de asa, principalmente a la furosemida y aunque estas proteínas

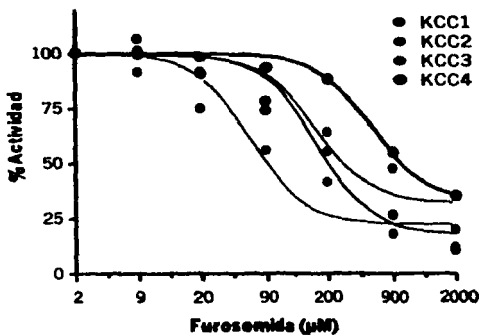


Figura 15. Curva dose-respuesta de inhibición por furosemida en los KCCs.

muestran alrededor del 22% de identidad con los cotransportadores cloro-catiónicos dependientes de Na^+ pueden ser inhibidos también por diuréticos del tipo de las tiazidas (datos no mostrados). Harting y colaboradores (132)

mostraron que el cotransportador AXI 4 cloro-catiónico de la planta del tabaco, que tiene mayor homología en secuencia con los KCCs (36-38%), también puede inhibirse por bumetanida, furosemida y diuréticos del tipo de las tiazidas como la metolazona.

Los KCCs, como muestra la figura 16, también pueden bloquearse por la adición de 10 mM de BaCl_2 en el medio de captación, con la siguiente sensibilidad relativa: $\text{KCC4} > \text{KCC3} > \text{KCC2} > \text{KCC1}$.

Ésta es la primera demostración de que los cotransportadores de $\text{K}^+:\text{Cl}^-$ clonados son sensibles al BaCl_2 y también concuerda con la propuesta controversial de Greger y Schlatter a principios de los 80's (56) de que las células de la membrana basolateral de la porción gruesa del asa ascendente de Henle expresan un cotransportador de $\text{K}^+:\text{Cl}^-$ sensible al bario. La observación hecha por Amal y colaboradores (57) de que el mecanismo de transporte de NH_4^+ sensible al bario y dependiente de Cl^- es sólo ligeramente

sensible a la furosemida, sugiere que el KCC4 es la isoforma presente en la membrana basolateral del asa ascendente de Henle en los mamíferos.

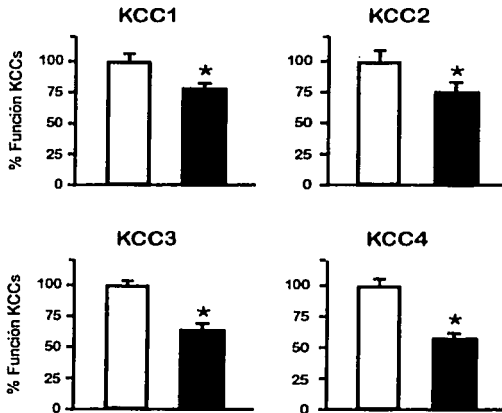


Figura 16. Efecto de 10 mM de BaCl₂ sobre la función de los KCCs. Las barras blancas representan a los grupos control y las barras negras los grupos tratados con BaCl₂. *p<0.01 vs la captación en el grupo control.

La inhibición de proteínas fosfatasa previene la activación inducida por NEM o hinchamiento celular del cotransportador de K⁺:Cl⁻ en algunas células (38, 42-44, 46) y se acepta que para la activación del transportador se requiere de su desfosforilación. De acuerdo con otros laboratorios, en el presente estudio se observó que el inhibidor de proteínas fosfatasa, calcicolina A, previene la activación hipotónica de los KCCs. La calcicolina A inhibe dos tipos de fosfatasa: PP1 y PP2A (133). Con el objeto de determinar el tipo de fosfatasa involucrada, se estudió además el efecto del ácido okadaico, que a una concentración de 1 nM inhibe específicamente a la PP2A y el efecto de la

cipermetrina que inhibe específicamente a PP2B (134, 135). Como se observa en la figura 17, a excepción de la caliculina A, ninguno de estos inhibidores, afectó la activación del cotransportador por hipotonicidad, lo cual sugiere que la PP1 es la fosfatasa involucrada en la activación de los KCCs durante el aumento del volumen celular (38, 43).

Inicialmente el cotransportador de K^+Cl^- fue definido como una vía de transporte de K^+ en eritrocitos activables con NEM. Muchos investigadores han encontrado que el pretratamiento de los eritrocitos de algunas especies con 1 mM de NEM resulta en una activación significativa de esta vía de transporte de K^+ dependiente de Cl^- (7).

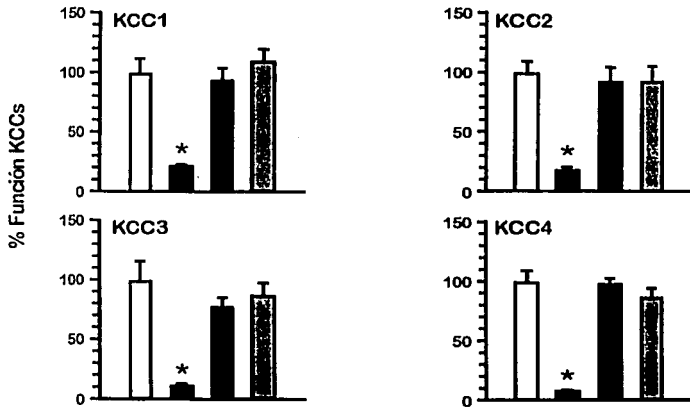


Figura 17. Efecto de inhibidores de proteínas fosfatasa sobre la función de los KCCs durante exposición de los ovocitos a soluciones hipotónicas. Barras blancas, control; barras negras, 100 nM caliculina A; barras rayadas, 100 pM cipermetrina. * $p < 0.01$ vs grupo control.

Aún no está claro si el efecto activador del NEM está relacionado con la defosforilación que induce a través de la activación de una cinasa localizada *cuesta arriba* en la cascada de señalización o por modificación directa de los grupos tiol en el cotransportador. Existen reportes que apoyan ambas posibilidades (136). Sin embargo, al igual que la activación por aumento del volumen celular, el transporte activado por NEM puede prevenirse por inhibidores de fosfatasas, sugiriendo un efecto positivo del NEM sobre las vías de señalización *cuesta arriba* (38, 42, 44, 49, 137). A este respecto, nuestros datos muestran un comportamiento muy interesante de los KCCs cuando se exponen a NEM. En la figura 18 se observa que en isotonicidad, el NEM estimula la función del KCC1, KCC3a y KCC4, así como al cotransportador endógeno de K^+Cl^- en los ovocitos (más adelante se muestran los resultados del cotransportador endógeno). Su y colaboradores también observaron la activación de KCC1 por NEM en ovocitos (98).

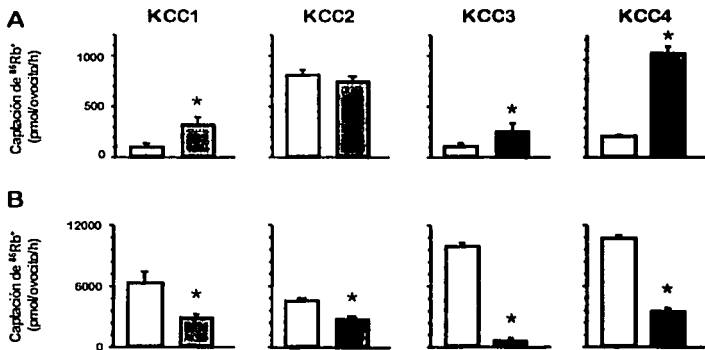


Figura 18. Efecto de la N-etilmaleimida sobre la función de los KCCs en medio isotónico (panel A) o en medio hipotónico (panel B). Control en barras blancas; 1 mM NEM en barras grises. * $p < 0.01$ vs. grupo control.

En contraste, en medio hipotónico, cuando los ovocitos fueron expuestos a NEM, todas las isoformas se inhibieron. El hecho de que los KCC1, 3a y 4 fuesen activados por NEM en medio isotónico sugiere que los ovocitos poseen las vías de señalización intracelular que el NEM requiere para la activación de los cotransportadores. Los mecanismos por los cuales estos KCCs, activados por hinchamiento, se inhiben por NEM se desconocen. En eritrocitos de oveja los cotransportadores de K^+Cl^- pueden ser activados o inhibidos por NEM mediante estimuladores de tioles de alta y baja afinidad, respectivamente (40, 138). Es necesario esclarecer este punto en futuros experimentos. Sin embargo, una propuesta que conjunta éstas y previas observaciones es que hay sitios de inhibición directa sobre los transportadores y sitios de estimulación en las cinasas *cuesta arriba* en la cascada de señalización. A este respecto, existen algunas cisteínas transmembranales o yuxtamembranales en las proteínas predichas para cada isoforma de KCCs y las cisteínas transmembranales se han implicado en la sensibilidad diferencial de los cotransportadores de $Na^+K^+2Cl^-$ a compuestos que reaccionan con cisteínas (139).

Se sabe también que algunos transportadores pueden afectarse por exposición a $HgCl_2$. Por ejemplo, el transporte a través de los cotransportadores de Na^+ /sulfuro (NaSi) (140) y de Na^+ /fosforo (NaPi-3) en ovocitos de *Xenopus* se reduce significativamente cuando los ovocitos se exponen a $HgCl_2$ (141). En contraste, la acuaporina 6 (AQP-6) es activada por este metal pesado cuando se expresa en ovocitos de *Xenopus* (142). Se sabe también que el Hg^{2+} afecta la función de canales y transportadores debido a que interactúa con grupos sulfhidrilo (-SH) o residuos de cisteínas (142, 143) y

en algunos casos esto se ha corroborado por estudios de mutagénesis (144, 145).

En la figura 19 se muestra que la exposición de ovocitos de *Xenopus* a 50 μM de HgCl_2 en medio hipotónico, resultó en una disminución de la actividad de KCC2 y KCC4 (50 ± 8.01 y 53.2 ± 4.18 %, respectivamente $p < 0.0001$), mientras que no tuvo efecto sobre la actividad de KCC1 y KCC3a (100.3 ± 2.68 y 93.6 ± 4.07 %, respectivamente $p = \text{NS}$). El mecanismo por el cual el Hg^{2+} afecta la función de las proteínas de membrana no está claro, pero se sabe interactúa con los grupos sulfhidrilo (-SH), y este estudio sugiere que la interacción del Hg^{2+} con los grupos sulfhidrilo es necesaria para el efecto inhibitorio.

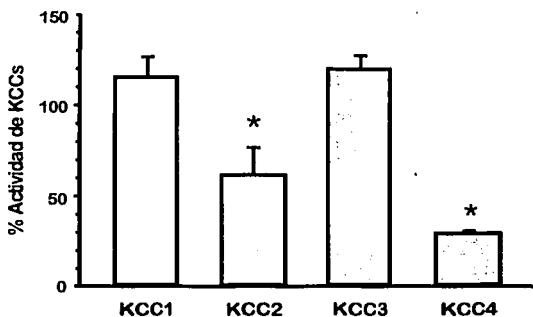


Figura 19. Efecto de 50 μM de HgCl_2 sobre la actividad de los KCCs en condiciones hipotónicas. * $p < 0.0001$ vs KCC1 o KCC3

Tabla 5. Propiedades funcionales de las isoformas del cotransportador de K⁺:Cl⁻

	KCCx	KCC1	KCC2	KCC3a	KCC3b	KCC4
Función en isotonicidad	Si	No	Si	No	No	No
Activación en hipotonicidad	+	++	+	+++	+++	++++
Inhibición por Calciclina A	Si	Si	Si	Si	Si	Si
Inhibición por A. Okadaico	No	No	No	No	No	No
Inhibición por Cipermetrina	No	No	No	No	No	No
Km K⁺ [mM]	27.7 ± 3.0	25.5±3.9	9.3±1.0	51.9±3.9	46.0±7.9	17.5±2.7
Km Cl⁻ [mM]	15.4 ± 4.7	17.2±8.3	6.8±0.9	14.2±3.1	11.1±5.2	16.1±4.2
Furosemide IC₅₀ [µM]	~ 200	~ 180	~ 70	~ 180	~ 220	~ 700
Inhibición por BaCl₂	+	+	+	++		++
Inhibición por HgCl₂	-	-	++	-	-	++
Serie de Aniones	Cl>PO ₄ >Br	Cl>SCN>Br	Cl>Br>I	Br>Cl>PO ₄	Br>Cl>PO ₄	Cl>Br>PO ₄
Localización intrarrenal		G, TP,TD	Ninguna		TP, VS	VS, TP, AH
Polarización		Apical			Basolateral	Basolateral

Abreviaciones: Cl= cloro; SCN= tiocianato; Br= bromuro; PO₄= fosfato; G=glomerulo; TP= túbulo proximal; TD= túbulo distal; VS= vasos sanguíneos; AH= asa de Henle.

El cotransportador de K^+Cl^- de los ovocitos de *Xenopus laevis*

Durante el transcurso de este estudio, además de caracterizar las isoformas cerebral y renales del cotransportador de K^+Cl^- , también obtuvimos datos suficientes que demuestran que los ovocitos de *Xenopus laevis* expresan un cotransportador endógeno de K^+Cl^- que denominamos KCCx. Este transportador tiene similitudes funcionales y moleculares con los KCCs de mamífero. La caracterización de KCCx fue fundamental debido a que hizo posible diferenciar la expresión del transportador endógeno de los exógenos. Aunque cabe mencionar que la expresión del cotransportador es muchas veces mayor en los ovocitos inyectados con el RNAc de los KCCS heterólogos que en los no inyectados.

La caracterización funcional se determinó a partir de ovocitos defoliculados de *Xenopus*, obtenidos por cirugía de ranas hembra adultas. Cuatro días después de defoliculados, los ovocitos se incubaron, en grupos de 15, por 30 minutos en medio hipotónico (~ 120 mOsm/kg) que nominalmente carecía de K^+ y Cl^- . Este medio tenía la siguiente composición en mM: 50 N-metil-D-glucamina (NMDG)-gluconato, 4.6 Ca^{2+} -gluconato, 1 Mg^{2+} -gluconato, 5 HEPES/Tris, pH 7.4 y 1 mM de ouabaina. A continuación los ovocitos se incubaron durante 60 minutos, para medir la captación de $^{86}Rb^+$, en un medio hipotónico que contenía concentraciones variables K^+ y Cl^- [en mM: 0-50 NMDG-Cl, 0-50 KCl, 0-50 NMDG-gluconato, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4], suplementado con 1 mM ouabaina y 2.5 $\mu Ci/ml$ de $^{86}Rb^+$. Al final del período de captación los ovocitos se lavaron en la solución de captación fría (sin el isótopo), para remover las trazas extracelulares del mismo, y uno a uno se colocaron en un tubo de centelleo líquido que contenía SDS al 10 % para

medir la captación del material radioactivo en un contador de centelleo β . Algunos experimentos también se realizaron en medio isotónico usando las mismas soluciones pero suplementadas con 3.5 g/100ml de sacarosa para aumentar la tonicidad de la solución a las condiciones isosmolares de los ovocitos (~ 210 mOsm/kg).

La caracterización molecular se realizó mediante la búsqueda de ESTs en la base de datos del GenBank, que mostró una secuencia EST de 549 pb (GenBank No de acceso AW646505) perteneciente a ovocitos de *Xenopus laevis* y con homología a los KCCs de mamífero. Posteriormente amplificamos dicho fragmento a partir del RNA total de ovocitos de *Xenopus* mediante RT-PCR. Los primers, sentido 5'-ACAGTACTTCTGGGAGACTACC-3' y antisentido 5'-GATACGTAATGATAAAGAAAGG-3', se diseñaron a partir de la secuencia del EST de *Xenopus* y amplificaron un fragmento de 528 pb. Para incrementar la especificidad de la PCR generamos dos primers internos, sentido 5'-AGCAGAGCAAGGCACTGAAACAC-3' y antisentido 5'-GGAAGGGCAGAAGCATAAGC-3', que amplificaron una banda de 359 pb.

El RNA total se extrajo a partir de ovocitos defoliculados y otros tejidos usando el método de homogeneización con isotiocianato de guanidina y ultracentrifugación en gradiente de cloruro de cesio. La PCR se realizó con 1 μ g de RT en 20 μ l de reacción y 35 ciclos de la siguiente manera: 1 min, 94°C; 1 min, 60°C y 1 min, 72°C. El último ciclo consistió de 5 min a 72°C como un paso de extensión final. El producto de PCR de los ovocitos se secuenció y una vez confirmado como un KCC de *Xenopus* homólogo al de mamíferos, se realizó un análisis de Southern blot a partir de todos los productos de PCR obtenidos de los tejidos de la rana bajo condiciones de alta estringencia y

usando el fragmento de 528 pb para generar una sonda no radioactiva. La hibridación de las bandas se detectó mediante la reacción de inmunoperoxidasa.

RESULTADOS Y DISCUSION

Como ya se mencionó, las células animales han desarrollado una serie de mecanismos reguladores del volumen celular tales como la DRVC que contrarrestan el hinchamiento osmótico producido por soluciones hipotónicas. Dicha respuesta reguladora se efectúa por la liberación de osmolitos intracelulares entre los que se encuentran los iones K^+ y Cl^- . Una de las vías celulares de flujo de dichos iones es el cotransportador de $K^+:Cl^-$. En ésta tesis se describen algunas propiedades funcionales de las isoformas conocidas de éste transportador (ver tabla 5), utilizando para ello un sistema de expresión heteróloga que tiene la ventaja única de permitir el análisis comparativo de las distintas isoformas en el mismo tipo celular: los ovocitos de *Xenopus laevis*. Este sistema ha sido utilizado exitosamente como herramienta de clonación y caracterización de otros transportadores y canales. En el presente trabajo encontramos evidencia de que los ovocitos tienen un cotransportador nativo de $K^+:Cl^-$.

La figura 20 muestra un resumen de 7 experimentos en los cuales la captación de $^{86}Rb^+$ fue probada usando una solución de captación que contenía 2 mM de K^+ y 50 mM de Cl^- externo. Las captaciones se realizaron en medios isotónicos e hipotónicos. Como muestra el panel A de la figura 20, en medio isotónico hubo una pequeña captación de $^{86}Rb^+$ de 10.1 ± 1.4 pmol/ovocito/h, que se redujo a 6.29 ± 1.3 pmol/ovocito/h cuando éstos fueron incubados en un medio en ausencia de Cl^- . La captación en presencia de Cl^-

externo aumentó a 52.5 ± 11.0 pmol/ovocito/h cuando los ovocitos se incubaron en medio hipotónico ($p < 0.00001$). El incremento en la captación de Rb^+ fue dependiente de Cl^- ya que la captación en medio hipotónico en ausencia de Cl^- fue 5.22 ± 1.0 pmol/ovocito/h ($p < 0.001$). Estos resultados concuerdan con los reportados por Lytle y McManus (146) quienes proponen que en ausencia de Na^+ externo, como es el caso de los experimentos realizados en éste trabajo, el cotransportador de $Na^+:K^+:2Cl^-$ puede catalizar el intercambio de K^+/K^+ sensible a bumetanida. En éstas circunstancias, es poco probable que la actividad del transporte de $^{86}Rb^+$ en ausencia de Na^+ se deba al cotransportador de

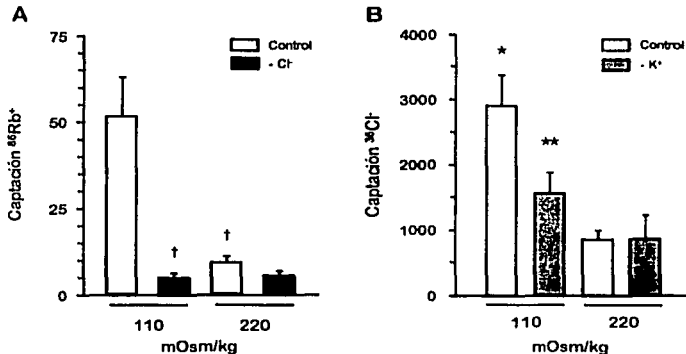


Figura 20. Efecto de la tonicidad del medio externo sobre la captación de $^{86}Rb^+$ (panel A) y $^{36}Cl^-$ (panel B) en ovocitos desfolículos de *Xenopus laevis*. * $p < 0.01$ vs control en 220 mOsm/kg, ** $p < 0.01$ vs control en 110 mOsm/kg y † $p < 0.001$ vs control en 110 mOsm/kg.

$Na^+:K^+:2Cl^-$, ya que éste transportador en los ovocitos es inhibido por la hipotonicidad del medio (123). Por lo tanto, los ovocitos presentan un mecanismo de captación de $^{86}Rb^+$ dependiente de Cl^- en medio isotónico, que puede ser activado por aumento del volumen celular. Así mismo, el panel B

también muestra el resultado de un experimento en el cual probamos la captación de $^{36}\text{Cl}^-$ en presencia de 10 mM de K^+ y 50 mM de Cl^- externo. En isotonicidad, la captación de $^{36}\text{Cl}^-$ fue similar en presencia o ausencia de K^+ externo (908 ± 124 vs 912 ± 346 pmol/ovocito/h, $p=\text{NS}$), mientras que la incubación en hipotonicidad indujo un incremento en la captación de $^{36}\text{Cl}^-$ de $2,960 \pm 455$ pmol/ovocito/h ($p<0.01$) que fue parcial pero significativamente dependiente de K^+ ($1,615 \pm 296$ pmol/ovocito/h [$p<0.01$]). Nuevamente podemos confirmar que los ovocitos también presentan un mecanismo de captación de $^{36}\text{Cl}^-$ dependiente de K^+ que es aparente sólo durante aumento del volumen celular.

El cotransporte de $\text{K}^+:\text{Cl}^-$ en algunas células muestra la propiedad única de ser activado por el agente alquilante N-etilmaleimida (NEM) (7). Nosotros analizamos el efecto del NEM sobre la captación de $^{86}\text{Rb}^+$ en ovocitos incubados en condiciones isotónicas o hipotónicas. La figura 21 muestra que

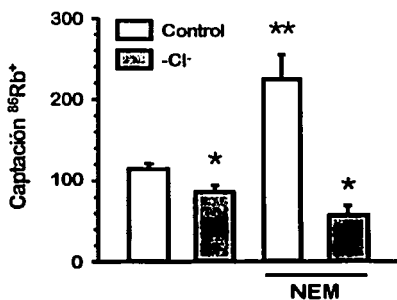


Figura 21. Efecto del NEM sobre la función del cotransportador de $\text{K}^+:\text{Cl}^-$ endógeno en medio isotónico. Los ovocitos fueron expuestos a la droga 15 min antes del período de captación.

durante isotonicidad, la captación de $^{86}\text{Rb}^+$ aumentó ~ 2 veces de 117 ± 6.3 a 227 ± 29 pmol/ovocito/h ($p<0.001$), en los ovocitos expuestos a 1mM de NEM antes del período de captación. El aumento en la captación inducida por NEM fue dependiente de Cl^- . En

ausencia de Cl^- externo la captación fue similar entre los ovocitos control y los tratados con NEM (88 ± 7 vs. 60 ± 11 pmol/ovocito/h, $p = \text{NS}$). Por lo tanto, la adición de NEM resultó en un aumento en la actividad del cotransportador de $\text{K}^+:\text{Cl}^-$ en medio isotónico.

El transporte electroneutro de K^+ y Cl^- es una de las vías de eflujo de iones que es activada durante el aumento de volumen celular, además de participar en la disminución reguladora del volumen celular (DRVC). La figura 22 muestra la activación del cotransportador de $\text{K}^+:\text{Cl}^-$ en ovocitos comprobada al medir el eflujo de $^{86}\text{Rb}^+$ cuando los ovocitos son expuestos a hipotonicidad. Durante un periodo de 150 minutos se observa una progresiva reducción de la cantidad de $^{86}\text{Rb}^+$ en las células (panel A, círculos blancos), junto con un incremento gradual en la cantidad de Rb^+ del medio externo (panel B, círculos blancos). Como muestran los círculos rellenos de la figura 22, la adición del diurético furosemda, significativamente redujo el eflujo de $^{86}\text{Rb}^+$ de las células,

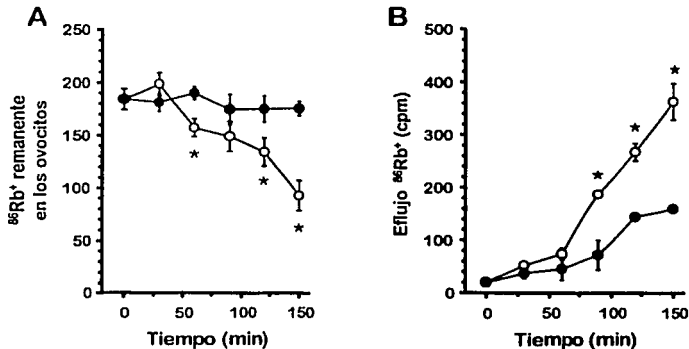


Figura 22. Eflujo de $^{86}\text{Rb}^+$ en ovocitos de *Xenopus laevis* en medio hipotónico. El panel A muestra la cantidad de Rb^+ restante en los ovocitos y el panel B la aparición del Rb^+ en el medio externo, ambos en función del tiempo y en ausencia, (círculos blancos) o presencia, (círculos rellenos) de furosemda. * $p < 0.01$ vs. los mismos tiempos con furosemda.

indicando la proporción del eflujo llevada a cabo por el cotransportador de K^+Cl^- en los ovocitos.

La figura 23 muestra la dependencia de K^+ externo (panel superior) y la dependencia de Cl^- externo (panel inferior) del cotransportador de K^+Cl^- en ovocitos de *Xenopus*. Las captaciones se realizaron en un medio externo con osmolaridad de 110 mOsm/kg, con una concentración fija de K^+ (20 mM) o Cl^- (50 mM), cambiando las concentraciones del contraion desde 0 hasta 50 mM. Como se observa en la figura 23, la captación de Rb^+ aumentó conforme la

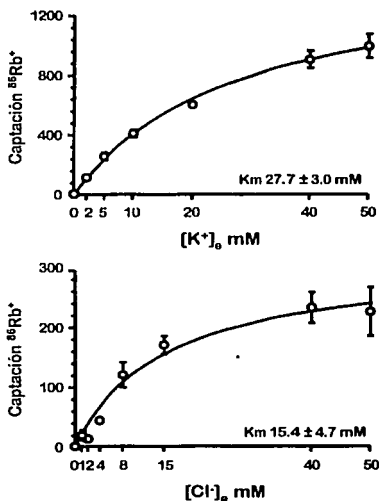


Figura 23. Análisis cinético de la captación de $^{86}Rb^+$ en ovocitos de *Xenopus*. Panel superior, captación de Rb^+ dependiente de K^+ externo y panel inferior, captación de Rb^+ dependiente de Cl^- externo.

concentración de cada ión transportado se incrementaba mostrando un comportamiento Michaelis-Menten. Las K_m y V_{max} aparentes calculadas para K^+ externo fueron 27.7 ± 3.0 mM y $1,531 \pm 78$ pmol/ovocito/h, respectivamente, y los valores de K_m y V_{max} para Cl^- externo fueron 15.4 ± 4.7 mM y 318 ± 39 pmol/ovocito/h, respectivamente. Consistente con la estequiometría de 1:1 del proceso de transporte, el coeficiente de Hill para ambos

iones está cerca de la unidad: 1.04 ± 0.17 y 1.07 ± 0.14 para K^+ y Cl^- , respectivamente.

Se ha mostrado que algunos aniones extracelulares además del Cl^- pueden ser translocados a través del cotransportador de $K^+:Cl^-$ en eritrocitos humanos y de oveja (147). Por lo tanto fue interesante estudiar la captación de $^{86}Rb^+$ en presencia de aniones diferentes al Cl^- para el cotransportador de $K^+:Cl^-$ en ovocitos desfolliculados. La figura 24 no muestra diferencias significativas entre la captación de $^{86}Rb^+$ en presencia de Cl^- , PO_4^- o Br^- (129 ± 18 ; 130 ± 21 y 101 ± 16 pmol/ovocito/h, respectivamente), mientras que hubo una reducción significativa en presencia de I^- , SCN^- y gluconato (67 ± 15 ; 44 ± 12 y 25 ± 5 pmol/ovocito/h, respectivamente. $P < 0.001$). De tal manera que el perfil de selectividad aniónica del cotransportador endógeno de $K^+:Cl^-$ fue $Cl^- = PO_4^- = Br^- > I^- > SCN^- =$ gluconato.

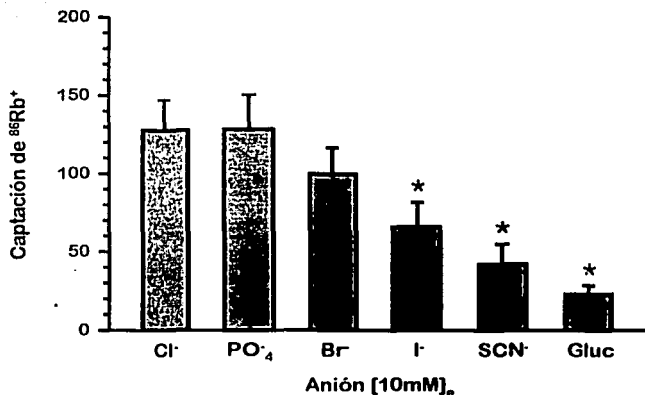


Figura 24. Perfil de selectividad aniónica del cotransportador endógeno de $K^+:Cl^-$ en ovocitos de *Xenopus*. * $p < 0.001$ vs. Cl^- .

La figura 25 muestra el efecto de algunos inhibidores de los cotransportadores electroneutros en la función del KCCx. La adición de 100 μM de furosemida o bumetanida al medio resultan en un 22 y 20 % de reducción en la captación de Rb^+ dependiente de Cl^- respectivamente, cuando se comparó con la captación del grupo control ($p < 0.05$). También se probó el efecto de otros inhibidores

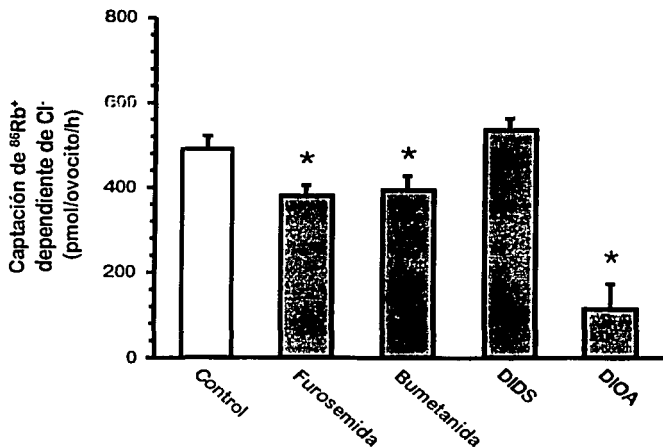


Figura 25. Efecto de algunos inhibidores del transporte sobre la captación de $^{86}\text{Rb}^+$ dependiente de Cl^- en ovocitos de *Xenopus* en medio hipotónico. * $p < 0.05$ vs. control.

conocidos sobre el cotransportador de $\text{K}^+:\text{Cl}^-$. La adición de 100 μM de DIDS no tuvo efecto significativo sobre la captación de Rb^+ , mientras que los KCCs de mamífero son sensibles a esta concentración de DIDS (7, 114, 126). En contraste, 100 μM de DIOA redujo la captación de Rb^+ 76 % ($p < 0.000001$). De modo que el KCCx es más sensible a DIOA que a los diuréticos de asa, como es el caso de las isoformas de mamífero (126). La figura 26 muestra la inhibición dependiente de la concentración por diuréticos de asa del

cotransportador de K^+Cl^- nativo de los ovocitos. El IC_{50} para furosemida y bumetanida se calculó en 200 y 500 μM , respectivamente. Aunque los cuatro KCCs de mamíferos difieren en la sensibilidad relativa a los dos diuréticos de asa, la bumetanida es siempre menos efectiva que la furosemida, como es en el caso del transportador endógeno.

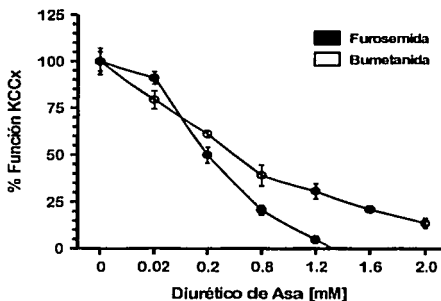


Figura 26. Curva de inhibición del cotransportador endógeno de K^+Cl^- por diuréticos de asa.

Se ha sugerido que los cotransportadores de K^+Cl^- también puedan inhibirse por bario (56). Por tal motivo decidimos probar el efecto de 10 mM de bario externo sobre la función del KCCx. El $BaCl_2$ redujo la captación a través del cotransportador. La

captación de $^{86}Rb^+$ dependiente de Cl^- en los ovocitos control fue de 135 ± 21 pmol/ovocito/h y se redujo a 62 ± 14 pmol/ovocito/h en presencia de $BaCl_2$. Ésta reducción del 45% fue estadísticamente significativa ($p < 0.01$).

En algunas células los cotransportadores de K^+Cl^- se activan por aumento del volumen celular a través de un mecanismo que incluye un incremento en la capacidad de desfosforilación de la célula. Por tal motivo se ha postulado que la desfosforilación del cotransportador puede formar parte de su mecanismo de activación (34). Considerando lo anterior, probamos el efecto de reducir en los ovocitos la capacidad de desfosforilación del cotransportador

de K^+Cl^- inhibiendo la función de las proteínas fosfatasa PP1, PP2A y PP2B con 100 nM de calcicolina A, 1 nM de ácido okadaico y 100 pM de cipermetrina, respectivamente. Como muestra la figura 27, el aumento en la captación de $^{86}Rb^+$ inducida por la hipotonicidad fue completamente bloqueada por calcicolina A. En contraste, no se observó ningún efecto en presencia de ácido okadaico o cipermetrina.

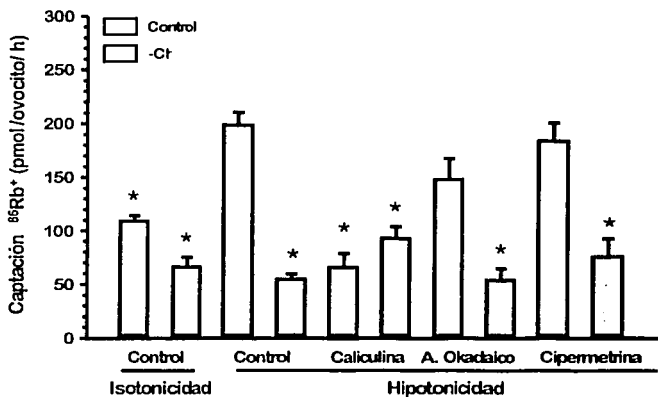


Figura 27. Efecto de la inhibición de proteínas fosfatasa sobre la activación del KCCx inducida por aumento del volumen celular en ovocitos de *Xenopus*. La captación se realizó en medio isotónico o hipotónico exponiendo a los ovocitos a calcicolina A, ácido okadaico o cipermetrina durante los períodos de precaptación y captación. La captación se realizó en un medio que contenía 10 mM de K^+ en presencia (barras blancas) o ausencia (barras grises) de Cl^- externo. * $p < 0.001$ vs. control en medio hipotónico.

Recientemente se ha demostrado que la función de algunos cotransportadores se reduce por su exposición a Hg^{2+} (139-141), mientras que otras proteínas, como la aquaporina 6 (AQP6), son estimuladas por este metal pesado (142). Por lo tanto, analizamos el efecto del Hg^{2+} sobre la captación de

$^{86}\text{Rb}^+$ en ovocitos de *Xenopus*. Como muestra la figura 28, la incubación de los ovocitos en presencia de $150 \mu\text{M}$ de HgCl_2 , en medio isotónico durante 15 minutos previos al periodo de captación resultó en un incremento importante en la captación de $^{86}\text{Rb}^+$. Este incremento fue completamente prevenido con un pretratamiento de los ovocitos con 10 mM del agente reductor ditioneitol (DTT) (139). La incubación de los ovocitos con y sin Cl^- externo durante el periodo de captación reveló que el Hg^{2+} induce un incremento en la captación de $^{86}\text{Rb}^+$ compuesto al menos por dos vías diferentes, cada una contribuyendo en $\sim 50\%$ de la captación total. Una de ellas, dependiente de Cl^- , que concuerda con la activación del cotransportador endógeno de $\text{K}^+:\text{Cl}^-$ y la otra, independiente de Cl^- que sugiere la apertura de un canal de K^+ .

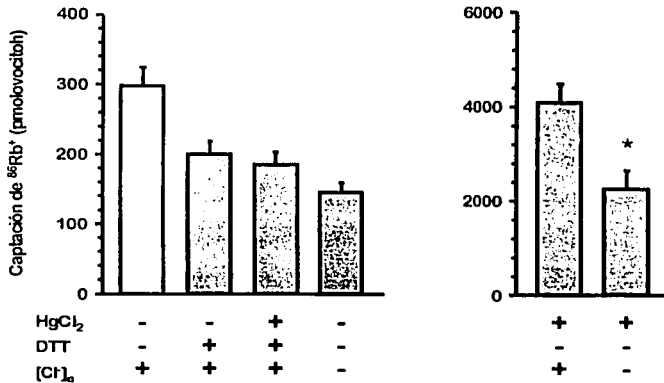


Figura 28. Efecto de $150 \mu\text{M}$ de HgCl_2 y 10 mM de ditioneitol (DTT) sobre la captación de $^{86}\text{Rb}^+$ en ovocitos de *Xenopus laevis*. Los ovocitos se incubaron en medio isotónico durante la captación. Antes de la captación los ovocitos se expusieron por 30 min al DTT solo, 15 min al HgCl_2 solo o 30 min al DTT más HgCl_2 en los últimos 15 min; el HgCl_2 y el DTT no estuvieron presentes durante el periodo de captación. Como se indica, la captación se llevó a cabo en presencia o ausencia de Cl^- externo. Cada punto representa la $\bar{X} \pm \text{EE}$ de un grupo de 30 ovocitos. * $p < 0.001$ vs. control con HgCl_2

Los resultados presentados hasta aquí definen las principales características funcionales, farmacológicas y probables vías de regulación del cotransporte de K^+Cl^- endógeno de los ovocitos de *Xenopus laevis*. Sin embargo, no se ha definido la naturaleza molecular de la proteína. Para ello, se realizó una búsqueda de secuencias homólogas a los KCCs de mamífero en la base de datos de los ESTs del GenBank. En ella se encontró un EST proveniente de ovocitos de *Xenopus laevis* (No. de acceso AW646505) con significativa homología a las secuencias de los KCCs en mamíferos. Dicha secuencia mostró ser 76% idéntica en secuencia al KCC1 de rata. Considerando lo anterior, diseñamos un par de primers a partir de la secuencia del EST, con los cuales se amplificó un fragmento de 528 pb, usando la reacción de transcripción reversa (TR) del RNA total extraído de diferentes órganos de *Xenopus*, incluyendo ovocitos. El panel superior de la figura 29 muestra una banda única del tamaño esperado (528 pb), amplificada de cada TR, excepto para el RNA de ovocito en ausencia de enzima (TR-). Posteriormente, el uso de un segundo par de primers internos que amplificaban una banda de 349 pb y la secuenciación de dichas bandas, confirmaron la naturaleza molecular del producto de PCR. La alineación de las secuencias de aminoácidos deducidas para cada KCC de mamífero con la secuencia parcial para el KCC de *Xenopus*, mostró que dicho fragmento COOH terminal es 68% idéntico al KCC1 de la rata, 56% al KCC2 de rata, 76% al KCC3 humano y 62% al KCC4 del ratón. Además de lo anterior, el panel inferior de la figura 28 muestra el análisis de Southern blot de los productos de RT-PCR de los órganos obtenidos, usando el fragmento de 528 pb como sonda no radioactiva.

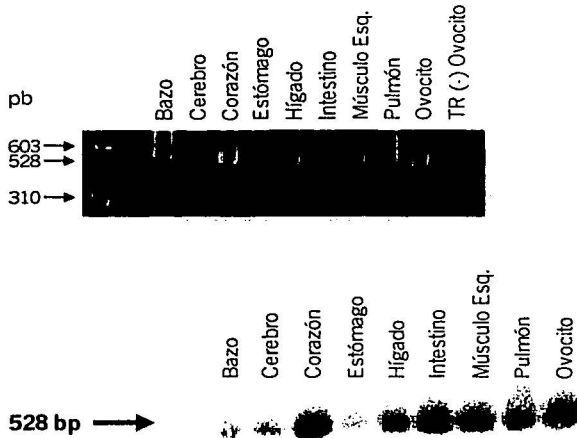


Figura 29. Análisis de RT-PCR (panel superior) y Southern blot (panel inferior) para la identificación del KCCx en diversos órganos de *Xenopus laevis*.

COMENTARIOS FINALES Y CONCLUSIONES

Para empezar a entender la fisiología y las relaciones estructura-función de los KCCs individuales, este trabajo tuvo como objetivo realizar en detalle el análisis funcional de las cuatro isoformas de mamífero de los KCCs empleando los ovocitos de *Xenopus laevis* como sistema de expresión. Así mismo fue posible reunir evidencia suficiente para sugerir que los ovocitos de *Xenopus* expresan un cotransportador de K^+Cl^- endógeno.

Este trabajo reveló diferencias significativas en cuanto a regulación, cinética y farmacología entre las todas las isoformas estudiadas. Estas diferencias podrían ser explicadas al comparar sus secuencias de aminoácidos. En general, la homología entre las cuatro proteínas KCCs de mamífero es de ~ 68 %, mientras que aquella entre los KCCs y otros cotransportadores cloro catiónicos es ~ 30 %. El análisis filogenético de todos los miembros de la superfamilia de los cotransportadores cloro-catiónicos (CCC) de mamífero indica que están organizados en dos grupos; el primero integrado por los cotransportadores de $Na^+K^+2Cl^-$ (CSB/NKCC) y de Na^+Cl^- (CST/NCC) y el segundo por los cuatro cotransportadores de K^+Cl^- (KCC).

Al comparar las secuencias de los KCCs encontramos que la isoforma KCC4 del humano comparte 69 % de identidad con el KCC2 de la rata, 65 % con el KCC1 del humano y 66 % con el KCC3 del humano. Mientras que la secuencia KCC3 del humano comparte 71 % de identidad con el KCC1 del humano, 66 % con el KCC2 de la rata y 66 % con el KCC4 del humano. De manera que la filogenia de estas proteínas sugiera que el grupo formado por los KCCs esté compuesto a su vez por dos subgrupos, KCC1/KCC3 y KCC2/KCC4 (ver figura 9).

Además de las diferencias funcionales, la selectividad de los iones transportados y la sensibilidad a diuréticos de cada grupo de la superfamilia de los CCC, la principal característica estructural distintiva entre los KCCs y los transportadores que unen Na^+ es la posición del asa extracelular. Dicha estructura se localiza entre los STM 5 y 6 en los KCCs y entre los STM 7 y 8 en CSB/NKCC y CST/NCC.

El porcentaje de identidad en la secuencia primaria es mayor entre los STM, seguida por el extremo COOH terminal y el NH_2 terminal que está ligeramente conservado (~77%, ~72% y ~44%, respectivamente). Dentro del dominio de los STM el grupo de Forbush ha mostrado que la afinidad a iones y diuréticos del cotransportador basolateral de $\text{Na}^+:\text{K}^+:\text{2Cl}^-$ (CSB2/NKCC1) está determinada por los residuos de aminoácidos que se localizan dentro de los STM 2, 4 y 7 (148, 149). Sin embargo, entre los KCCs, los STM 2, 4, 5 y 7 son los menos conservados (42, 61, 52 y 62%, respectivamente) por lo que es posible sugerir que la variación en estos segmentos determina no sólo las diferencias cinéticas, sino la afinidad a diuréticos. Las diferencias en las secuencias entre los dominios intracelulares NH_2 y COOH también pueden explicar la activación variable por aumento del volumen celular entre los miembros de esta subfamilia de transportadores. Una gran inserción en el extremo carboxi terminal de KCC2 contiene un sitio potencial de fosforilación de tirosina (150) que está ausente en KCC1-3-4, aunque la región equivalente de KCC4 también contiene un sitio potencial de fosforilación para tirosina. El extremo NH_2 terminal de hKCC3a es más largo que cualquiera de los otros KCCs y contiene un gran número de sitios potenciales para fosforilación por PKC. La secuencia completa de hKCC3a indica un total de 13 sitios para PKC y 2 para PKA, mientras que la

secuencia del hKCC4 incluye 9 sitios para PKC, ninguno para PKA y un sitio para fosforilación de tirosina.

En conclusión, la comparación funcional y estructural entre los KCCs pone de manifiesto que, aunque estos transportadores difieren en algunas características importantes, incluyendo la respuesta al cambio en el volumen celular y la selectividad aniónica, todas comparten las siguientes características: 1) un cotransporte de $K^+ : Cl^-$ activado por aumento del volumen celular inducido en medio hipotónico, 2) un cotransporte de $K^+ : Cl^-$ activable con el agente alquilante NEM, 3) la activación del transporte por aumento del volumen celular, requiere de un evento de desfosforilación, 4) el cotransporte, puede inhibirse con caliculina A, un inhibidor específico de la PP1A.

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Cloning and Characterization of KCC3 and KCC4, New Members of the Cation-Chloride Cotransporter Gene Family*

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The K⁺-Cl⁻ cotransporters (KCCs) belong to the gene family of electroneutral cation-chloride cotransporters, which also includes two bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporters and a thiazide-sensitive Na⁺-Cl⁻ cotransporter. We have cloned cDNAs encoding mouse KCC3, human KCC3, and human KCC4, three new members of this gene family. The KCC3 and KCC4 cDNAs predict proteins of 1083 and 1160 amino acids, respectively. The KCC3 and KCC4 proteins are 65–71% identical to the previously characterized transporters KCC1 and KCC2, with which they share a predicted membrane topology. The four KCC proteins differ at amino acid residues within key transmembrane domains and in the distribution of putative phosphorylation sites within the amino- and carboxyl-terminal cytoplasmic domains. The expression of mouse KCC3 in *Xenopus laevis* oocytes reveals the expected functional characteristics of a K⁺-Cl⁻ cotransporter: Cl⁻-dependent uptake of ⁸⁶Rb⁺ which is strongly activated by cell swelling and weakly sensitive to furosemide. A direct functional comparison of mouse KCC3 to rabbit KCC1 indicates that KCC3 has a much greater volume sensitivity. The human KCC3 and KCC4 genes are located on chromosomes 5p15 and 15q14, respectively. Although widely expressed, KCC3 transcripts are the most abundant in heart and kidney, and KCC4 is expressed in muscle, brain, lung, heart, and kidney. The unexpected molecular heterogeneity of K⁺-Cl⁻ cotransport has implications for the physiology and pathophysiology of a number of tissues.

Potassium chloride cotransporters (KCCs)¹ were first de-

scribed as a swelling-activated K⁺ efflux in erythrocytes, the cell type for which functional characterization is the most complete (1, 2). The cotransport of K⁺ and Cl⁻ in erythrocytes is interdependent, with a 1:1 stoichiometry and low affinity constants for both ions (2). KCCs are not influenced by membrane potential, and under most physiological conditions, they function as an efflux pathway. K⁺-Cl⁻ cotransport is activated by hypotonicity and functions in regulatory volume decrease (1, 2). K⁺-Cl⁻ cotransport is sensitive to the loop diuretics bumetanide and furosemide, albeit with much lower drug affinities than Na⁺-K⁺-2Cl⁻ cotransport (3, 4). A number of other important characteristics serve to distinguish K⁺-Cl⁻ cotransport from Na⁺-K⁺-2Cl⁻ cotransport, in particular the response to protein phosphorylation and dephosphorylation (1).

A major impediment to the study of K⁺-Cl⁻ cotransport has been the lack of specific high affinity inhibitors. Convincing evidence for K⁺-Cl⁻ cotransport has nonetheless been reported for a number of tissues, including epithelia (5, 6), heart (7, 8), skeletal muscle (9), and brain (4, 10). In addition to cell volume regulation, non-erythroid K⁺-Cl⁻ cotransport has been implicated in trans-epithelial salt absorption (5, 11), renal K⁺ secretion (12, 13), and the regulation of both intra- and extracellular K⁺-Cl⁻ (4, 9, 10, 14).

Full-length cDNAs encoding two K⁺-Cl⁻ cotransporters, KCC1 and KCC2, were recently reported (3, 4, 16–17). Both proteins are homologous to the other electroneutral cation-chloride cotransporters, the bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporters BSC1 and BSC2 (also known as NKCC2 and NKCC1, respectively), and the thiazide-sensitive Na⁺-Cl⁻ cotransporter TSC (also known as NCC) (18, 19). Heterologous expression of KCC1 in HEK-293 cells results in Cl⁻-dependent, furosemide-sensitive uptake of ⁸⁶Rb⁺ that is activated by cell swelling (3, 16). KCC1 transcript is abundant in both mouse and human erythroleukemia cell lines, indicating that KCC1 is the major erythroid K⁺-Cl⁻ cotransporter (17). The widespread expression of KCC1 also suggests a significant role in non-erythroid K⁺-Cl⁻ cotransport (3, 16, 20). In contrast to KCC1, the expression of KCC2 is restricted to neurons within the central nervous system (15, 21). Along with other neuronal pathways for chloride (21, 22), KCC2 plays an important role in the regulation of the transmembrane chloride gradient and thus affects the neuronal response to stimuli such as γ -aminobutyric acid (14, 23).

An increasing amount of data suggest further heterogeneity in the proteins that mediate K⁺-Cl⁻ cotransport. At the func-

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[†] The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[®]™/EBI Data Bank with accession number(s) AF087436 (mKCC3), AF105365 (hKCC3), and AF105366 (hKCC4).

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[¶] The abbreviations used are: KCC, K⁺-Cl⁻ cotransporter; BSC or NKCC, bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter; TSC or NCC, thiazide-sensitive Na⁺-Cl⁻ cotransporter; h, human (e.g. hKCC); m, mouse (e.g. mKCC); ⁸⁶Rb⁺, rubidium; EST, expressed sequence tag; bp, base pair(s); kb, kilobase (or kilobase pair); IMAGE, integrated

molecular analysis of genomes and their expression; TIGR, The Institute for Genome Research; UTR, untranslated region; RT-PCR, reverse transcriptase polymerase chain reaction; RACE, rapid amplification of conserved ends; VNTR, variable number of tandem repeats; TM, transmembrane segment; PKC, protein kinase C.

TABLE I
 Oligonucleotide primers for PCR

Name	Sequence	Start	Clone
S1	5'-GGAGGCAGATAATCCCTTTCTCC-3'	2472	mKCC3
S2	5'-GATCTTCCCGACTACATCTTCC-3'	738	mKCC3
S3	5'-CCATCTAAATACGACTCACTATAGGC-3'		AP1 adaptor
S4	5'-GACTCTTTTCCGCAAAAGC-3'	2488	hKCC3
S5	5'-CGAAGTAACATGACACTTTTCG-3'	200	hKCC3
S6	5'-ATCCCA1GCCCACGCAACTTTIACCG-3'	69	mKCC3
S7	5'-AGTCAGAATCCATCACAGGG-3'	441	hKCC4
S8	5'-CTATCTTCCGATCTGACC-3'	1723	hKCC4
S9	5'-CACAGATTCACCCCTGTGACG-3'	4241	hKCC4
S10	5'-CTATATACCTCTTTCGCTTTCG-3'	3624	hKCC4
S11	5'-CACTCTGACGATCAAGGG-3'	3858	hKCC4
AS1	5'-CACAGCATATGCATCTTCC-3'	3135	mKCC3
AS2	5'-ACACTCTCTCGAGTCCAGG-3'	2187	mKCC3
AS3	5'-ACCGCCATGAGTCCGACGGCCAGCTGC-3'	863	mKCC3
AS4	5'-TTCCTTAGAAGCCACCGGATGTCAAGT-3'	1007	mKCC3
AS5	5'-AGACTGCTGTGTCGTTGTGG-3'	3280	hKCC3
AS6	5'-CAGGCGCATGACACCACTG-3'	786	hKCC3
AS7	5'-GAAA GCGGCTCACATGTAC-3'	513	hKCC3
AS8	5'-CCTCGAAAATGTCCCATATGCTGG-3'	2889	hKCC4
AS9	5'-GAAGGCGCAATAACAATACC-3'	1895	hKCC4
AS10	5'-CGAGCAGTCTCATCGAAC-3'	2821	hKCC4
AS11	5'-GCAACCTCTTATGGACACC-3'	4618	hKCC4
AS12	5'-GCAGCATGTTTCAAGTATGATCC-3'	4185	hKCC4

tional level, KCC1 and KCC2 differ in K^+ affinities (3, 4) ($K_m > 2.5$ and 5.2 mM, respectively), and a dramatically higher K^+ affinity (K_m , 0.9 mM) has also been reported for insulin-stimulated K^+ -Cl⁻ cotransport in a mouse muscle cell line (9). Erythroid K^+ -Cl⁻ cotransport is heterogeneous with respect to inhibition by the K^+ -channel blocker quinidine, with a significant quinidine-insensitive fraction of Cl⁻-dependent K^+ flux (24). At the molecular level, cDNA probes that include human KCC1 coding sequences detect a number of transcripts, indicative of either alternative splicing or the expression of closely related gene products. Thus, a rat 3'-UTR probe detects transcripts of 3.8 and 4.4 kb in size (3), and hKCC1 probes from within the open reading frame also detect transcripts of 5.5 (20, 25) and 6–7 kb (3) in various human tissues and cell lines. Using the mouse and human EST data bases as a starting point, we have cloned cDNAs corresponding to two of these new KCC isoforms, denoted KCC3 and KCC4. Functional expression in *Xenopus laevis* oocytes confirms that mouse KCC3 is a furosemide-sensitive K^+ -Cl⁻ cotransporter, with much greater volume sensitivity than rabbit KCC1. Although highly homologous, KCCs 1–4 differ in potentially important transmembrane (TM) segments and in the distribution of putative cytoplasmic phosphorylation sites. Northern blot analysis with specific 3'-UTR probes indicates that the KCCs have distinct but overlapping expression patterns. The identification of all four KCCs thus reveals significant differences in structure, function, and expression.

MATERIALS AND METHODS

cDNA Cloning

mKCC3—A BLAST (basic alignment and search tool) search (26) of the EST data base revealed a number of mouse ESTs that were homologous to rat KCC1 and KCC2. The cDNAs corresponding to four of these ESTs (IMAGE clones 568084, 633794, 313521, and 659105) were obtained from the IMAGE consortium (Research Genetics, Genome Systems and/or the ATCC) and sequenced. These ESTs contained partial open reading frames homologous to various segments of KCC1 and KCC2 (Fig. 1). The 3'-end of the open reading frame and the entire 3'-UTR were identified in a 608 bp IMAGE cDNA (clone 1314578). The tissue distribution of mKCC3 was then assessed by RT-PCR, and widespread expression of mKCC3 was detected with the S1/AS1 primer pair (Table I), with particularly abundant transcripts in kidney and heart (data not shown). Using a primer pair (S2 and AS2 primers, Table I) spanning the gap between the IMAGE clones 313521 and 568084, a 1.2-kb fragment was amplified from C57BL/6J kidneys and subcloned

into the *EcoRV* site of pBluescript by blunt-end ligation. PCR conditions for these and other gene-specific primers were optimized using Taq 2000 and the Opti-Prime buffer system (Stratagene). The following amplification protocol was followed, unless specified otherwise: 30 cycles of denaturation (92 °C, 2 min), annealing (64 °C, 1 min), and extension (72 °C, 1 min), followed by a final extension step (72 °C, 8 min). The extreme 5'-end of mKCC3 was cloned from DAL3c mouse kidney 5'-RACE template (CLONTECH), using two antisense primers (primers AS3 and AS4, Table I) and the AP1 adaptor primer from CLONTECH (S3 primer). This PCR utilized AmpliTaq-Gold DNA polymerase (Perkin-Elmer) and a hot-start amplification protocol, consisting of a 9-min enzyme activation step at 94 °C, followed by 35 cycles of 94 °C for 1 min and 68 °C for 2.5 min, and a final 10-min extension at 72 °C.

KCC3—A human KCC EST clone (TIGR clone 150738) was obtained from the ATCC. Sequencing revealed that this cDNA is derived from the human ortholog of mKCC3. Another human 5'-EST (GenBank[®] accession number F12342) exhibited strong amino acid homology with the extreme 3' coding sequence of the other KCCs. This EST overlaps with a large number of ESTs from the 3'-UTR of hKCC3, including the IMAGE clones 22250 and 51311 (Fig. 1). The gap between these cDNAs and the TIGR clone 150738 (Fig. 1) was bridged by RT-PCR with the primer pair S4/AS5 (Table I), using a human kidney template (CLONTECH). The PCR products for this reaction were subcloned into pBluescript. Finally, the 5'-end of hKCC3 was amplified by sequential RT-PCR of human kidney with the primer pairs S6/AS6 and S6/AS7. S6 and S6 were derived from IMAGE clones 1403108 and mKCC3, respectively.

KCC4—Sequence analysis of another human EST cDNA (TIGR clone 150620) indicated the existence of a fourth KCC, hKCC4. Northern blot analysis revealed significant expression in muscle, and a single 5'-RACE cDNA was cloned from a human muscle template (CLONTECH) using the S3/AS8 primer pair. This 5'-RACE PCR used Advantage polymerase mix (CLONTECH) and a hot-start protocol consisting of the following: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 3 min. Screening a random-primed human muscle cDNA library (CLONTECH) with a probe encompassing nucleotides 741–871 of hKCC4 yielded a single cDNA that extended 5' of the start codon. Finally, two overlapping PCR fragments were amplified from human brain template (CLONTECH) with the S7/AS9 and S8/AS10 primer pairs (Table I), and subcloned into pCR2.1 by TA cloning (Invitrogen).

Sequence Analysis of KCC3 and KCC4 cDNAs

All cDNA clones were sequenced on both strands using fluorescent dye terminator chemistry (Applied Biosystems). For cDNA sequence derived exclusively from PCR, at least two cDNAs from two separate PCR reactions were sequenced. Analyses of the nucleotide and amino acid sequences were performed using the GeneWorks 2.6 and MacVec-

for 6.5 software packages (Oxford Molecular). Alignments and other analyses also made use of the computer programs BLAST (26), DNAS-*nr*, and SMART (simple modular architecture research tool) (27).

Chromosomal Localization and Genomic Structure of hKCC3 and hKCC4

Chromosomal assignments for the human KCC3 and KCC4 genes were made using a PCR-based screening approach with the National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel 1 (28). The primers used for hKCC3 mapping (59 and AS11) amplify a 377-bp segment of the 3' noncoding region from genomic DNA, and the hKCC4 mapping primers (S10 and AS12) amplify a 661-bp segment of the 3' noncoding sequence. PCR reactions using DNA from the NIGMS panel were scored for the presence or absence of the appropriately sized product using agarose gel electrophoresis.

The chromosomal localization of hKCC3 was verified by sequencing the chromosome 6 genomic clone pMS621, a gift of Dr. John Armour (see "Results"). Fine mapping of hKCC4 was performed by PCR using radiation hybrid analysis with the Stanford C3 panel (Research Genetics) and the S10AS12 primer pair. Reaction products generated by PCR were alkaline-denatured, applied to a nylon membrane using a dot-blot apparatus, and subjected to Southern blotting with a ³²P-labeled internal oligonucleotide probe (primer S11, Table I). Results were analyzed by querying the Stanford radiation hybrid map (<http://www.shgc.stanford.edu/rlh/>).

Northern Blots and RT-PCR

RNA was extracted from mouse tissues (C57BL/6J strain) using guanidine isothiocyanate and cesium chloride. Total RNA (10 μg/ml) was size-fractionated by electrophoresis (5% formaldehyde, 1% agarose), transferred to a nylon membrane (Stratagene), and probed sequentially with ³²P-labeled randomly primed probes corresponding to full-length glyceraldehyde-3-phosphate dehydrogenase and nucleotides 4417-5062 of mKCC3 (3'-UTR). Human multiple-tissue Northern blots containing 2 μg/lane poly(A)⁺ RNA (CLONTECH) were hybridized to probes generated by PCR from the 3'-UTRs of hKCC3 (nucleotides 4598-4857) and hKCC4 (nucleotides 3624-4185) and to a human β-actin probe. Hybridization for all blots was performed overnight at 42 °C in 4 × SSCP, 40% formamide, 4 × Denhardt's solution, 0.5% SDS, and 200 μg of salmon sperm DNA, and membranes were washed twice for 10 min at room temperature in 2 × SSCP, 0.1% SDS and twice for 1 h at 65 °C in 0.1 × SSCP, 0.1% SDS. Exposure times varied as noted in the legends of Figs. 4 and 5.

In Vitro Translation of mKCC3 Protein

1.0 μg of the full-length mKCC3 cDNA (see below) was translated *in vitro* using [³⁵S]methionine and T7 RNA polymerase-coupled rabbit reticulocyte lysate (TNT[™] T7, Promega), both with and without pancreatic microsomes, for 90 min at 30 °C. Protein was resolved by 7% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

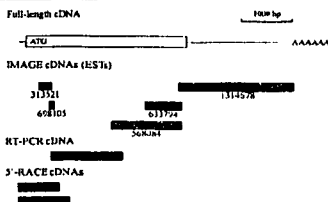
Expression of mKCC3 in *X. laevis* Oocytes

For functional expression and *in vitro* translation, a full-length mKCC3 cDNA was assembled in the *Xenopus* expression vector pGEMHE (29). The resulting construct contains nucleotides 55-3812 of mKCC3. For functional comparison with mKCC3, a full-length rabKCC1 cDNA, the gift of Dr. Bilsa Forbush III, was subcloned into pGEMHE. To prepare a template for cRNA, the rabbit KCC1 and mKCC3 cDNAs were linearized at the 3'-end using *Xba*I, and cRNA was transcribed *in vitro* using the T7 RNA polymerase and the mMES-SAP6 RNA MACHINE kit (Ambion).

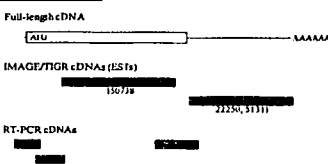
Defolliculated stage V-VI oocytes were injected with 60 nl of water or a solution containing cRNA at a concentration of 0.5 μg/μl (25 ng/oocyte). Oocytes were incubated at 17 °C in ND96 (96 mM Na⁺-Cl⁻, 2 mM K⁺-Cl⁻, 1.8 mM Ca²⁺-Cl⁻, 1.0 mM Mg²⁺-Cl⁻, and 5 mM Hepes/Tris, pH 7.4), supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml penicillin, for 3 days.

Functional expression of rabbit KCC1 and mKCC3 was assessed by measuring tracer ⁸⁶Rb⁺ uptake in groups of 20-25 oocytes 3 days after water or cRNA injection. Because K⁺-Cl⁻ cotransport is activated by cell swelling, both hypotonic and isotonic conditions were used. ⁸⁶Rb⁺ uptake was measured after a 30-min incubation period in either hypotonic or isotonic Na⁺-free and Cl⁻-free medium. Hypotonic medium (~125 mosmol/kg) contained 48 mM *N*-methyl-D-glucamine-Cl⁻, 2 mM K⁺-Cl⁻, 1.8 mM Ca²⁺-Cl⁻, 4.0 mM Cu²⁺-gluconate, 1.0 mM Mg²⁺-gluconate, and 6 mM Hepes/Tris, pH 7.4. To generate the isotonic conditions (~220 mosmol/kg) sucrose was added to a concentration of 100 mM. Ouabain

Mouse KCC3



Human KCC3



Human KCC4

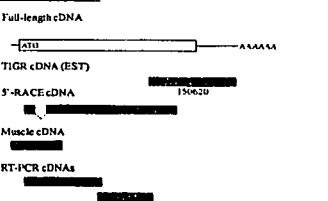


Fig. 1. Schematic representation of KCC3 and KCC4 cDNAs. A full-length cDNA is shown for each isoform. Coding sequences are boxed, and solid lines represent the 5' and 3'-UTR. The relative positions of partial cDNAs, derived from EST cDNA clones, RT-PCR, 5'-RACE RT-PCR, and library screening, are shown below each full-length sequence. The IMAGE or TIGR clone numbers are displayed below EST cDNAs. The figure is drawn to the scale indicated.

was added to both solutions at a concentration of 1 mM. This incubation period was followed by a 60-min uptake period using Na⁺-free uptake medium, both hypotonic and isotonic. Hypotonic uptake medium (~125 mosmol/kg) contained 48 mM *N*-methyl-D-glucamine-Cl⁻, 2 mM K⁺-Cl⁻, 1.8 mM Ca²⁺-Cl⁻, 1 mM Mg²⁺-Cl⁻, and 5 mM Hepes/Tris, pH 7.4. The isotonic conditions (~220 mosmol/kg) included 100 mM sucrose. Both uptake solutions contained 5.0 μCi/ml of ⁸⁶Rb⁺ (New England Nuclear) and 1 mM ouabain. To determine the Cl⁻-dependent fraction of ⁸⁶Rb⁺ uptake, pairs of groups of oocytes were incubated in uptake medium without Cl⁻ (substituted with gluconate). When indicated, 2 mM furosemide was added to both the incubation and uptake media. All uptake experiments were performed at 30 °C, after which oocytes were washed five times in ice-cold uptake solution without isotope to remove tracer activity in the extracellular fluid. After the oocytes were dissolved in 10% SDS, tracer activity was determined by β-scintillation counting.

RESULTS

Sequence of KCC3 and KCC4—Full-length KCC3 and KCC4 sequences (deposited in the GenBank[™] data base under ac-

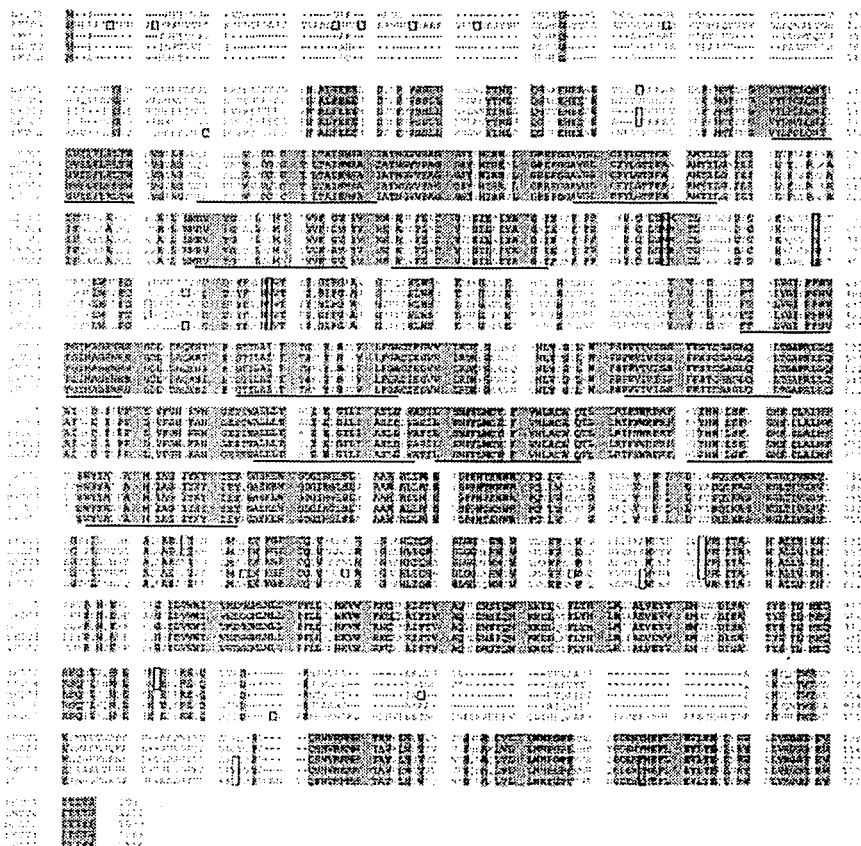


Fig. 2. Protein alignments of the four mammalian K^+-Cl^- cotransporters. The deduced amino acid sequences of mKCC3, hKCC3, and hKCC4 are aligned together along with hKCC1 and rat KCC2. Identical segments are boxed in grey, and putative TM segments are underlined in black. Consensus sites for N-linked glycosylation are boxed in green within the predicted extracellular loop between TM-6 and TM-7. Consensus phosphorylation sites for PKC are boxed in red, protein kinase A sites are in yellow, and tyrosine kinases are boxed in blue.

cession numbers AF087436 (mKCC3), AF105365 (hKCC3), and AF105366 (hKCC4) were determined from a number of overlapping cDNA clones (Fig. 1). The complete mouse and human KCC3 cDNAs are 5132 and 6230 nucleotides long, respectively, which is close in size to the KCC3 transcripts seen on Northern blots (Figs. 5A and 6A). Both cDNAs contain open reading frames of 3248 nucleotides, with 85% identity, and the predicted proteins contain of 1083 amino acids and exhibit 91%

identity. The KCC3 3'-UTRs are only 34% identical, close to the lower limit of conservation between mouse and human orthologs (30).

The hKCC4 cDNA sequence is 4237 nucleotides in length, with a 5'-UTR of 165 bp and a 3'-UTR of 622 bp. The discrepancy between the cDNA size and the size of the KCC4 transcripts (Fig. 6C), as well as the lack of a consensus polyadenylation site within the 3'-UTR, suggests that the 3'-UTR

sequence is incomplete. There are three in-frame start codons between nucleotides 165 and 195 of the hKCC4 cDNA, at which translational initiation would result in proteins of 1150, 1141, or 1135 amino acids in length. However, homology to KCC1 and KCC3 begins before the third methionine (Fig. 2), which is thus an unlikely translational start site. Comparison with the mouse KCC4 sequence² indicates significant conservation of the first nine codons, which also contain a PKC site (Fig. 2), and hence translation likely occurs at the first start codon.

At least two KCC4 transcripts of 6–7 kb are detected by Northern blot analysis (Fig. 6C), consistent with alternative splicing. In comparison with other KCC4 5' cDNAs (Fig. 1), the single 5'-RACE cDNA contained a deletion of nucleotides 708–854, encoding TM-1 and TM-2 in the predicted KCC4 protein. The deleted region corresponds precisely to exon 4 of hKCC1 (16), and hence at least part of the heterogeneity in KCC4 transcripts is the result of the alternative splicing of coding exons.

The four KCC proteins are 65–71% identical (Fig. 2). Sequence alignments indicate that hKCC3 shares 69% identity with rat KCC2, 65% identity with hKCC1, and 66% identity with hKCC4. The hKCC4 protein shares 71% identity with hKCC1, 66% identity with rat KCC2, and 66% identity with hKCC3. The identity between the KCCs and other cation-chloride cotransporters is in the range of 27 to 33%. A phylogenetic tree indicates that the mammalian cation-chloride cotransporters fall into two groups, one composed of the Na⁺-K⁺-2Cl⁻ cotransporters and the Na⁺-Cl⁻ cotransporter, and the other encompassing the four K⁺-Cl⁻ cotransporters. As indicated by direct sequence alignments, the four KCCs form two subgroups, KCC1 paired with KCC4 and KCC3 paired with KCC2 (Fig. 3).

The seven mammalian cation-chloride cotransporters share a predicted membrane topology. A central core of 12 TM domains is flanked by hydrophilic amino- and carboxyl-terminal domains that are known to have a cytoplasmic orientation (18). The major structural difference between the KCCs and the Na⁺-linked cotransporters is the position of a large glycosylated extracellular loop, which is predicted to occur between TM-5 and TM-6 in the KCCs and between TM-7 and TM-8 in the Na⁺-2Cl⁻ cotransporters and the Na⁺-Cl⁻ cotransporter (18). Homology is most marked in the TM domains, the intracellular loops, and the cytoplasmic carboxyl terminus (Fig. 2).

A comparison of the four KCCs reveals a number of intriguing differences. KCC4 is the longest of the four because of an extension of ~60 amino acids at the extreme amino-terminal end (Fig. 2). Although highly conserved, none of the TM domains in the four KCCs are completely identical. Within the cytoplasmic domains the four KCCs differ significantly in the distribution of consensus phosphorylation sites for tyrosine kinases, protein kinase A, and protein kinase C (PKC). A carboxyl-terminal tyrosine phosphorylation site in KCC2 (Tyr¹⁰⁸⁷) is conserved in mouse and human KCC3 (Tyr¹⁰⁸⁴) (Fig. 2). The KCC4 sequence predicts a total of 11 PKC sites, 7 contained within the first 90 amino acids (Fig. 2). KCC4 has two potential protein kinase A sites, one of which (Ser³³⁹) is a predicted substrate for both protein kinase A and C. The KCC3 sequences predict fewer PKC sites, of which only two are conserved in both mouse and human (Thr²¹⁴ and Ser²⁰⁶).

KCC1 (3), KCC2 (4), and other members of the cation-chloride cotransporter gene family (18) are known to be glycoproteins, and the four KCC sequences contain three identical N-linked glycosylation sites in the otherwise poorly conserved

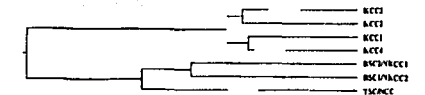


Fig. 3. Phylogenetic relationships between mammalian cation-chloride cotransporters. The six human cation-chloride cotransporters are compared with rat KCC3 in a phylogenetic tree generated with the DNASar program.

extracellular loops (Fig. 2). The *in vitro* translation of mKCC3 results in a protein with an apparent molecular mass of ~115 kDa, slightly lower than the predicted core weight of 119 kDa. The addition of canine pancreatic microsomes results in the appearance of an additional band of higher molecular mass (Fig. 5C), which is consistent with *in vitro* glycosylation.

Chromosomal Localization of hKCC3 and hKCC4—Chromosomal assignments for human KCC3 and KCC4 were defined using a PCR-based somatic cell hybrid mapping strategy. The genes for KCC3 and KCC4 were assigned to chromosomes 5 and 15, respectively. Fine mapping by radiation hybrid analysis places the hKCC4 gene on chromosome 15q14 between the markers D15S1040 and D15S118. Further localization of KCC3 was facilitated by the finding that D5S110 (31), a chromosome 5 VNTR (variable number of tandem repeats) minisatellite marker, is contained within the gene. The corresponding genomic subclone, pMS621 (the gift of Dr. John Armour), was sequenced to verify that it contained hKCC3 exons just 5' of the VNTR (Fig. 4). The exon/intron boundaries in the hKCC1 gene (16) are conserved in this portion of the hKCC3 gene, and the exons in pMS621 correspond to exons 14–17 of hKCC1 (Table II). The chromosome 5 summary map generated by the Wessex Human Genetics Institute (<http://cedar.genetics.soton.ac.uk/pub/chrom5/map.html>) indicates that D5S110 is on 5p15.3, between D5S678 and the telomere.

Tissue Distribution of KCC3 and KCC4—Northern blot analysis was performed with probes derived from the 3'-UTRs of KCC3 and KCC4. KCC3 probes detect 5.3-kb transcripts in a number of tissues, most prominently in the heart and kidney (Figs. 5A and 6A). Very little KCC3 transcript is detectable in adult brain. KCC4 has a more restricted expression pattern, with significant amounts of transcript found only in muscle, brain, lung, heart, and kidney (Fig. 6C). At least two different transcripts of 6–7 kb hybridize to KCC4 probes, consistent with alternative splicing (see above).

Functional Expression of mKCC3 and Rabbit KCC1—Microinjection of *Xenopus* oocytes with cRNA *in vitro* transcribed from mKCC3 and rabbit KCC1 resulted in significant increases in ⁸⁶Rb⁺ uptake compared with water-injected oocytes. The increased ⁸⁶Rb⁺ uptake was evident only when oocytes were incubated in hypotonic medium. Fig. 7A shows ⁸⁶Rb⁺ uptake in H₂O, rabbit KCC1, and mKCC3-injected oocytes, using isotonic and hypotonic conditions. In H₂O-injected oocytes ⁸⁶Rb⁺ uptake increased from 26.3 ± 5.33 pmol/oocyte/h under isotonic conditions to 46.8 ± 2.79 pmol/oocyte/h in hypotonic conditions (*p* < 0.01). In hypotonic medium, however, tracer Rb⁺ uptake decreased to 4.2 ± 0.43 pmol/oocyte/h in the absence of extracellular Cl⁻ (*p* < 0.001) and to 17.9 ± 1.6 pmol/oocyte/h in the presence of 2 mM furosemide, suggesting the presence of an endogenous K⁺-Cl⁻ cotransporter in *Xenopus* oocytes. In rabbit KCC1-injected oocytes, ⁸⁶Rb⁺ uptake increased from 8.8 ± 0.5 pmol/oocyte/h in isotonic medium to 200 ± 13.8 in hypotonic medium (*p* < 0.001). The observed uptake was Cl⁻-dependent and furosemide-sensitive. ⁸⁶Rb⁺ uptake in mKCC3-injected oocytes increased from 15.3 ± 1.4 pmol/oocyte/h when oocytes were incubated in isotonic conditions (~220 mosmol/liter) to

² D. B. Mount and E. Delprat, unpublished data.

pMS621

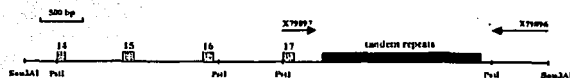


Fig. 4. Structure of the pMS621 genomic subclone. The pMS621 clone contains exons 14–17 of the hKCC3 gene, which flank the D6S110 VNTR. The polymorphic tandem repeat, 1.8 kb in length in the pMS621 clone, can vary between 0.5 and 10 kb (31). Arrows and GenBank™/EBI accession numbers indicate the previously reported DNA sequence flanking the repeat.

TABLE II
Exon/Intron boundaries of *KCC3* exons 14–17

Exon	Length	Acceptor site	Donor site	Intron
14	100 bp	tccctgcagGTCTTCCT	CTACCCTGgtgaggcta	643bp
16	119 bp	cgccccagGACCCGTG	GTACCCCGgttaagccc	659bp
16	104 bp	tccctcacagGCCCAAGAA	GACCTGGAGgtgagcct	650bp
17	169 bp	ttgctgcagGCCCAAGT	CGCCGAGGAGtggcgcc	

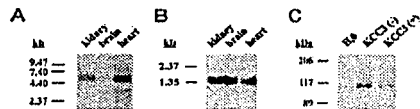


Fig. 5. *A*, Northern blot analysis of mKCC3 (48-h exposure). The transcript is abundant in kidney and heart and undetectable in brain. *B*, the same blot was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (2-h exposure). *C*, *In vitro* translation of mKCC3 protein. ³⁵S-labeled mKCC3 polypeptide was translated from cDNA by rabbit reticulocyte lysate, both with (+) and without (-) pancreatic microsomes, resolved by 7% SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography (12-h exposure).

2.552 ± 126 pmol/oocyte/h when incubated in hypotonic medium (~ 125 mosmol/liter). Most of the ⁸⁶Rb⁺ uptake observed in mKCC3-injected oocytes was Cl⁻-dependent, because uptake in mKCC3 oocytes in Cl⁻-free medium was 65 ± 5.8 pmol/oocyte/h. The activation of mKCC3 by cell swelling is significantly higher than that observed for H₂O-injected oocytes or for rabbit KCC1-injected oocytes. Fig. 7*B* shows that the Cl⁻-dependent fraction of ⁸⁶Rb⁺ uptake was 1.9 ± 0.1 -fold higher than the isotonic base line in control oocytes, 38.6 ± 2.8 -fold higher in rabbit KCC1-injected oocytes, and 226 ± 11.5 -fold higher than base line in oocytes injected with mKCC3.

DISCUSSION

We report here the cloning of KCC3 and KCC4, expanding the family of mammalian cation-chloride cotransporters to a total of seven members (Fig. 3). All of the known cation-chloride transporters belong functionally and/or structurally in one of three groups, encompassing two Na⁺-K⁺-2Cl⁻ cotransporters, one Na⁺-Cl⁻ cotransporter, and four K⁺-Cl⁻ cotransporters. A phylogenetic comparison of protein sequences indicates that the four K⁺-Cl⁻ cotransporters evolved separately from the Na⁺-dependent transporters, forming a distinct subfamily (Fig. 3). This separation is also evident at the genomic level, since the exon/intron structure of the BSC/NKCC genes (32, 33) is similar to that of the TSC/NCC gene (34), but differs significantly from that of the KCC1 gene (16). Partial characterizations of the mouse KCC2 gene (GenBank™ accession number AJ011033) and the human KCC3 gene (Table II) indicate that the KCCs have a conserved genomic structure.

The four KCC proteins share a 65–71% identity in primary structure. Within the central hydrophobic core, TM-2 and TM-4 show the most variation (Fig. 2); data from an extensive study

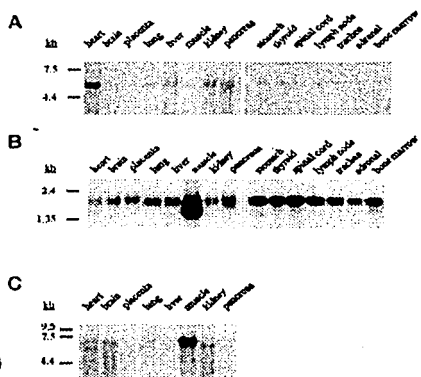


Fig. 6. Northern blot analysis of hKCC3. Human multiple-tissue Northern blots (CLONTECH) were probed with a cDNA probe from the 3'-UTR of hKCC3 (4-day exposure). *A*, A 5.3-kb transcript is seen in multiple tissues. *B*, the same Northern blots reprobed with a human β -actin probe (12-h exposure). *C*, Northern blot analysis of hKCC4, using a 3'-UTR probe (2-day exposure). Only one multiple-tissue blot was positive. Variants expression of two 6–7 kb transcripts is detected in muscle, heart, kidney, lung, and brain.

of ion affinity in the Na⁺-K⁺-2Cl⁻ cotransporters implicates TM-2 in cation transport (35) and TM-4 in anion transport (36). The cytoplasmic carboxyl terminal contain segments of substantial homology interspersed with a number of more variable regions. The extreme carboxyl terminus is identical in the four KCCs. Indeed, this homology was crucial in identifying ESTs that contained the 3'-end of hKCC3 (see "Materials and Methods"). Presumably some of the conserved cytoplasmic segments are involved in protein-protein interactions; however, none of the KCCs contain recognizable protein signaling domains (27). The predicted amino and carboxyl termini differ significantly in the distribution of putative phosphorylation sites (see Fig. 2 and "Results"). KCC4, in particular, is rich in potential PKC sites. The KCC2 and KCC3 sequences predict a tyrosine phosphorylation site near the extreme carboxyl terminus. This con-

sensus phosphorylation site is altered in KCC1 and KCC4 by variation in amino acids flanking the conserved tyrosine (Fig. 2).

The structural similarity between the four KCCs strongly suggests that both KCC3 and KCC4 encode K^+-Cl^- cotransporters. The functional expression of both KCC1 (3, 16) and KCC2 (4) has already been reported, using heterologous expression in HEK-293 cells. Using modification of a previously published protocol (37), we have utilized *X. laevis* oocytes for the functional expression of rabbit KCC1 and mKCC3. The significant identity between hKCC4 and the other KCCs suggests that it too encodes a K^+-Cl^- cotransporter; however, functional expression of this cDNA has not yet been examined. *Xenopus* oocytes have an endogenous bumetanide-sensitive $Na^+-K^+-2Cl^-$ cotransporter that is activated by hypertonicity and inhibited by hypotonicity (37). The activity of this cotransporter was abolished in the present study by the use of Na^+ -free conditions. The lack of extracellular Na^+ also exposes an endogenous K^+-Cl^- cotransporter that is only weakly activated by cell swelling (Fig. 7A), permitting a clear distinction between oocytes injected with KCC cRNA and controls injected with water. Oocytes injected with cRNA generated from the rabbit KCC1 and mKCC3 cDNAs express Cl^- -dependent uptake of $^{86}Rb^+$, which is partially sensitive to furosemide. A furosemide concentration of 2 mM inhibited the K^+-Cl^- cotransport activity of rabbit KCC1 and mKCC3 by 81 and 69%, respectively. Rabbit KCC1 and mKCC3 are both activated by cell swelling but differ markedly in the degree of activation over baseline isotonic conditions. Thus, rabbit KCC1 is activated 35-fold by hypotonic conditions, compared with the 226-fold activation of mKCC3 (Fig. 7B). Functional expression in HEK-293 cells suggests that rat KCC2 is insensitive to cell swelling (4). However, this KCC has not yet been compared with KCC1 and KCC3 using the *Xenopus* expression system. Although direct phosphorylation of KCC proteins has not been demonstrated, it is known that protein phosphorylation and dephosphorylation play important roles in the regulation of red cell K^+-Cl^- cotransport (1, 38). KCC1 and KCC3 differ in the distribution of putative phosphorylation sites for a number of protein kinases (Fig. 2), which may account in part for their differential sensitivity to cell swelling.

The 5.3-kb KCC3 transcript is widely expressed in both mouse and human, and ESTs have been reported from an array of cDNA libraries. However, in both species KCC3 transcript is most abundant in the heart, followed by the kidney. Cultured chick cardiac cells possess a K^+-Cl^- cotransporter capable of both inward and outward K^+-Cl^- transport (7). The role of this transport pathway in cardiac physiology is not yet clear. However, α_1 -adrenoreceptor stimulation of isolated rat hearts stimulates a K^+ efflux pathway that is partially sensitive to loop diuretics (39). The cell swelling induced by cardiac ischemia also stimulates a significant efflux of K^+ that is mediated predominantly by K^+-Cl^- cotransport. This efflux of K^+ may play an important role in the genesis of arrhythmias following myocardial ischemia (8).

KCC4 has a more restricted expression pattern than KCC1 and KCC3, confined primarily to muscle, brain, heart, and kidney. An insulin-stimulated K^+-Cl^- cotransport system with a high affinity for extracellular K^+ has been described in mouse skeletal muscle cells (9); however, the physiological roles of K^+-Cl^- cotransport in this tissue have not been extensively studied. KCC4 cDNAs were also cloned by RT-PCR from human brain (Fig. 1), in which a single transcript of ~6 kb has been detected by Northern analysis. Like KCC2, KCC4 may function in neuronal Cl^- homeostasis, with secondary effects on the response to γ -aminobutyric acid and other stimuli

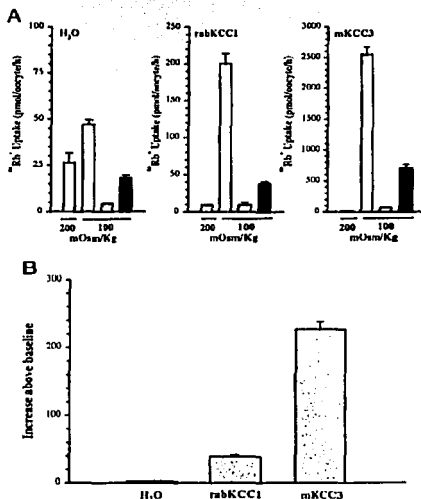


Fig. 7. Functional expression of mKCC3 and rabKCC1 in *X. laevis* oocytes. A, $^{86}Rb^+$ uptake in oocytes injected with H₂O, rabKCC1 or mKCC3. Uptakes were performed under both isotonic (200 mosmol/kg) and hypotonic (100 mosmol/kg) conditions in the presence (open bars) or absence (gray bars) of extracellular chloride and in the presence of 2 mM furosemide (black bars). There is a significant difference in the vertical scale between the three graphs. B, relative activation by hypotonicity of K^+-Cl^- cotransport expressed as a multiple of the respective uptakes in isotonic conditions. The endogenous K^+-Cl^- cotransporter (H₂O) exhibits only a 2-fold activation, compared with the 39-fold activation of rabbit KCC1 (rabKCC1) and a 226-fold activation of mKCC3.

(10, 14, 21, 23).

The combined results from this and other reports indicate that the mammalian kidney expresses KCC1 (3, 16), KCC3, and KCC4 (Figs. 5 and 6). There is strong physiological evidence for K^+-Cl^- cotransport in several nephron segments, including proximal tubule (5, 40), thick ascending limb (6, 11), distal convoluted tubule (12), and cortical collecting duct (13). The expression of three KCCs in the kidney suggests the possibility that there is an overlap in their intrarenal distribution. Moreover, one or more KCCs may target to the apical membrane of renal tubular cells, particularly within the cortical nephron (12, 13). The intrarenal distribution of hKCC1 was recently studied by *in situ* hybridization with a coding sequence probe (20). Although the probe chosen cross-hybridizes with KCC3, it is evident from this study that KCCs are expressed along the entire nephron, including within glomeruli (20). The role of K^+-Cl^- cotransport within the glomerulus is unknown. However, glomerular mesangial cells express BSC2/NKCC1 *in vivo* and exhibit hormone-sensitive $Na^+-K^+-2Cl^-$ cotransport (18). There is also preliminary evidence that cation-chloride cotransporters help set intracellular chloride activity in mesangial cells (41).

The human KCC3 gene has been localized to chromosome 5. Fine mapping to 5p15.3, between the anchor marker D5S678 and the telomere, was facilitated by the identity between a

segment of hKCC3 and sequence flanking the VNTR D5S110 (31). A genomic clone containing this VNTR also contains four exons of the hKCC3 gene (Fig. 4) homologous to exons 14–17 of the hKCC1 gene (Table II) (16). Polymorphism involving the D5S110 VNTR is generated by variable repetition of a core 11-bp sequence motif (31). The hKCC3 gene is not a particularly likely candidate for genetic disorders mapped to 5p15. However, KCC3 is expressed within the kidney, and it may play a role in basolateral ion transport within the thick ascending limb (6, 11). KCC3 is therefore a functional candidate gene for the remaining cases of Bartter's syndrome not caused by mutations in HSC1/NKCC2, ROMK, or CLC-NKB (42). In this regard, repeat expansion of a 12-bp motif within the cystatin B promoter has been shown to cause progressive myoclonic epilepsy (43), and expansion of the D5S110 VNTR could also conceivably affect the expression of hKCC3.

Human KCC3 has been localized to chromosome 16q14 between the markers D16S1040 and D16S118. This section of chromosome 15 has been linked to two subtypes of idiopathic generalized epilepsy (44, 45) and to a neurophysiological phenotype associated with schizophrenia (46). The markers D16S1040 and D16S118 also flank a 5-centimorgan region of chromosome 16q14 containing the gene for peripheral neuropathy with or without agenesis of the corpus callosum (Andermann's syndrome) (47).

In summary, we have cloned KCC3 and KCC4, two new members of the cation-chloride cotransporter family. The unexpected heterogeneity of K⁺-Cl⁻ cotransport raises a number of important issues for future study. First, the overlapping expression patterns of the four KCCs suggests the possibility that certain cell types will express more than one isoform, and indeed this appears to be the case in some human cell lines (25). Second, the significant homology within cytoplasmic domains has implications for the generation of isoform-specific antibodies. Third, the structural and functional comparison of the KCCs suggests that they differ in a number of important characteristics, including the response to changes in cellular volume (Fig. 7). Fourth, the full characterization of the physiological role(s) of individual KCCs will likely require sequential targeted disruption of the mouse KCC genes.

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Note Added in Proof—While this manuscript was in press, Hiki et al. published the characterization of human KCC3 (Hiki, K., D'Andrea, R. J., Furze, J., Crawford, J., Wellatt, E., Sutherland, G. R., Vindus, M. A., and Gamble, J. R. (1999) *J. Biol. Chem.* 274, 10661–10667). This KCC3 cDNA is an alternative splice form of the KCC4 cDNA reported here. In deference to the earlier publication, we have reversed the numbering of our GenBank™/EMBL submissions and will henceforth refer to the KCC on chromosome 16q14 as KCC3 and the KCC on chromosome 5p15 as KCC4.

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Functional Comparison of the K^+ - Cl^- Cotransporters KCC1 and KCC4*

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The K^+ - Cl^- cotransporters (KCCs) are members of the cation-chloride cotransporter gene family and fall into two phylogenetic subgroups: KCC2 paired with KCC4 and KCC1 paired with KCC3. We report a functional comparison in *Xenopus* oocytes of KCC1 and KCC4, widely expressed representatives of these two subgroups. KCC1 and KCC4 exhibit differential sensitivity to transport inhibitors, such that KCC4 is much less sensitive to bumetanide and furosemide. The efficiency of these anion inhibitors is critically dependent on the concentration of extracellular K^+ , with much higher inhibition in 50 mM K^+ versus 2 mM K^+ . KCC4 is also uniquely sensitive to 10 mM barium and to 2 mM trichloroethazine. Kinetic characterization reveals divergent affinities for K^+ (K_m values of ~25.5 and 17.5 mM for KCC1 and KCC4, respectively), probably due to variation within the second transmembrane segment. Although the two isoforms have equivalent affinities for Cl^- , they differ in the anion selectivity of K^+ transport ($Cl^- > SCN^- = Br^- > PO_4^{3-} > I^-$ for KCC1 and $Cl^- > Br^- > PO_4^{3-} = I^- > SCN^-$ for KCC4). Both KCCs express minimal K^+ - Cl^- cotransport under isotonic conditions, with significant activation by cell swelling under hypotonic conditions. The cysteine-alkylating agent *N*-ethylmaleimide activates K^+ - Cl^- cotransport in isotonic conditions but abrogates hypotonic activation, an unexpected dissociation of *N*-ethylmaleimide sensitivity and volume sensitivity. Although KCC4 is consistently more volume-sensitive, the hypotonic activation of both isoforms is critically dependent on protein phosphatase 1. Overall, the functional comparison of these cloned K^+ - Cl^- cotransporters reveals important functional, pharmacological, and kinetic differences with both physiological and mechanistic implications.

In a great majority of cells, the plasma membrane is permeable to water. Movement of water across the cell membrane is largely dependent on the osmotic pressure gradient between the intracellular and extracellular space, such that water

transport accompanies changes in the concentration of osmotically active molecules. Thus, when intracellular osmolality exceeds that of the extracellular milieu, cell volume increases due to the movement of water into the cell. To cope with the resultant cell swelling, cells have developed a series of complex mechanisms to achieve a regulatory volume decrease, primarily through the activation of efflux mechanisms for intracellular ions. In particular, net electroneutral release of K^+ - Cl^- is achieved by K^+ - Cl^- cotransport, the simultaneous operation of K^+/H^+ and Cl^-/HCO_3^- exchangers, or through parallel, swelling-activated K^+ and Cl^- channels (1).

K^+ - Cl^- cotransport was first described in red blood cells as a swelling- and NEM-activated K^+ efflux mechanism (2, 3), and red cells remain the primary model tissue for this class of ion transport. However, functional and physiological evidence has also been reported for the existence of K^+ - Cl^- cotransport in neurons (4), vascular smooth muscle (5), endothelium (6), epithelia (7, 8), heart (9), and skeletal muscle (10). Consequently, K^+ - Cl^- cotransport has been implicated not only in regulatory volume decrease, but also in transepithelial salt absorption (8), renal K^+ secretion (11), myocardial K^+ loss during ischemia (9), and regulation of neuronal Cl^- concentration (4). The physiological mechanisms invoked in cell volume regulation may also have broader roles in phenomena such as cell growth and apoptosis (1).

A major advance in the understanding of K^+ - Cl^- cotransport has been the recent molecular identification of mammalian genes that encode a total of four K^+ - Cl^- cotransporter (KCC) isoforms. These cotransporters were identified due to their similarity to other members of the electroneutral cation-chloride cotransporter gene family, the bumetanide-sensitive Na^+ - K^+ - $2Cl^-$ cotransporters and the thiazide-sensitive Na^+ - Cl^- cotransporter (12). The K^+ - Cl^- cotransporters have been designated KCC1 (13), KCC2 (14), KCC3 (15, 16), and KCC4 (16).³ KCC2 is a neuronal specific isoform, whereas the other three KCCs are widely distributed in multiple tissues. Phylogenetic and genomic analysis (16, 17) indicates that the four KCC

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¹ The abbreviations used are: NEM, *N*-ethylmaleimide; KCC, K^+ - Cl^- cotransporter; KCC4, mouse KCC4 isoform; KCC1, rabbit KCC1 isoform; ⁴⁵Rb⁺, tracer rubidium; TM, transmembrane segment; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DIOA, $R(+)$ -[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1-*H*-indenyl-5-yl)-oxy]acetic acid; HEK 293, human embryonic kidney cell line; BSC1/NKCC2, bumetanide-sensitive Na^+ - K^+ - $2Cl^-$ cotransporter 1 (renal specific); BSC2/NKCC1, bumetanide-sensitive Na^+ - K^+ - $2Cl^-$ cotransporter 2.

³ In our previous work (16), we referred to the KCC on human chromosome 16q14 as KCC4 and the KCC on chromosome 5p15 as KCC3. However, in deference to the earlier publication of Hiki et al., we reversed the numbering of our GenBank™/EMBL submissions to refer to the KCC on chromosome 16q14 as KCC3 and the KCC on chromosome 5p15 as KCC4 (see note added in proof in Ref. 16).

proteins form a separate subfamily of the cation-chloride cotransporters. Furthermore, KCC2 and KCC4 form a closely related subgroup, whereas KCC1 is more homologous to KCC3. Alternative splicing and alternative promoter usage generate further molecular heterogeneity. For example, there are at least two alternative isoforms of KCC3, generated by transcriptional initiation 5' of two separate first coding exons. The longer isoform, KCC3a (16), utilizes exon 1a, whereas KCC3b uses exon 1b, situated ~23 kilobases 3' within the human KCC3 gene on chromosome 10q14.2. The predicted KCC3a and KCC3b proteins, 1156 and 1099 amino acids, respectively, differ dramatically in the content and distribution of predicted phosphorylation sites for protein kinases.

The extent of molecular heterogeneity in K⁺-Cl⁻ cotransport was unexpected, even after the identification of KCC1 and KCC2. In consequence, next to nothing is known about the functional and pharmacological properties of the four major KCC isoforms, or indeed of the physiological role of each isoform. One exception is the recent recognition that KCC2 encodes a developmentally regulated Cl⁻ extrusion mechanism in neurons, with crucial secondary effects on the response to y-aminobutyric acid and other neurotransmitters that activate neuronal chloride conductance (4). To begin to understand the physiology and function of the individual KCCs, it is essential to characterize the functional properties of each isoform. KCC1 cDNAs from human, mouse, rabbit, pig, and *Caenorhabditis elegans* have been functionally expressed in human embryonic kidney cells (HEK 293) and in *Xenopus laevis* oocytes (13, 17, 18). Rat KCC2 and human KCC3 cDNAs have also been expressed in HEK 293 cells (15, 19, 20). From these studies, it is already quite clear that there are differences in the functional and pharmacological properties of the K⁺-Cl⁻ cotransporter isoforms. For example, kinetic analysis indicates that KCC2 exhibits significantly a higher affinity for potassium (14) than that of KCC1 (13) or KCC3 (20). In contrast, KCC2 is uniquely volume-insensitive, exhibiting minimal if any activation by cell swelling and considerable isosmotic transport activity (21). In the present study, we extended the functional and pharmacological characterization of the recently cloned mouse KCC4 (16), using the *Xenopus laevis* oocyte expression system. The functional comparison of shrike and human BSC2/NKCC1 and rabbit BSC1/NKCC2 has yielded important structure-function information for the Na⁺-K⁺-2Cl⁻ cotransporters (22–24). With this paradigm in mind, we simultaneously studied the functional properties of KCC1 in our expression system and report significant functional and pharmacological differences between these representatives of the two molecular subgroups of the KCCs.

MATERIALS AND METHODS

Xenopus laevis Oocyte Preparation.—Adult female *Xenopus laevis* frogs were purchased from Carolina Biological Supply Company (Burlington, NC) and maintained at the Institution animal facility under constant control of room temperature and humidity, 18 °C and 65%, respectively. Frogs were fed with frog brittle dry food from Carolina Biological Supply Company, and water was changed twice a week. Oocytes were surgically collected from anesthetized animals under 0.17% tricaine and incubated for 1 h with vigorous shaking in frog Ringer ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM Hepes, pH 7.4) in the presence of 2 mg/ml of collagenase B. Oocytes were then washed four times in ND96, manually defolliculated, and incubated overnight in ND96 at 18 °C. On the next day, stage V-VI oocytes (25) were injected with 50 nl of water containing 0.2–0.5 µg/µl of cRNA *in vitro* transcribed from mouse KCC4 (KCC4) or rabbit KCC1 (KCC1) cDNA. Oocytes were incubated at 18 °C for 4 days in ND96 supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml gentamicin; this incubation medium was changed every 24 h. On the day of

the experiment, oocytes were switched to Cl⁻-free ND96 (96 mM Na⁺-isothionate, 2 mM K⁺ gluconate, 6.0 mM Ca²⁺ gluconate, 1.0 mM Mg²⁺ gluconate, 5 mM Hepes, 2.5 mM sodium pyruvate, 5 mg/100 ml gentamicin, pH 7.4) 2 h prior to the uptake assay.

The full-length KCC4 and KCC1 cDNAs were previously subcloned into the high expression vector pGEMHE (16); rabbit KCC1 was a gift of Dr. Blaise Forbush III. To prepare cRNA, the KCC1 and KCC4 cDNAs were linearized at their 3' ends with *Xba*I and then transcribed *in vitro* using the T7 RNA polymerase mMESAGE kit (Ambion). Transcription product integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA). cRNA was stored frozen in aliquots at -80 °C until used.

Assessment of K⁺-Cl⁻ Cotransport—K⁺-Cl⁻ cotransport was assessed by measuring tracer ⁸⁶Rb⁺ uptake (NEN Life Science Products) in experimental groups of at least 15 oocytes. Since both KCC4 and KCC1 express minimal activity under isotonic conditions (see "Results"), ⁸⁶Rb⁺ uptake was generally assessed in oocytes swollen by a 30-min incubation period in a hypotonic K⁺ and Cl⁻-free medium (50 mM N-methyl-D-glucamine (NMDG) gluconate, 4.6 mM Ca²⁺ gluconate, 1.0 mM Mg²⁺ gluconate, 5 mM Hepes, pH 7.4) with 1 mM ouabain, followed by a 60-min uptake period in a hypotonic Na⁺-free medium with variable K⁺-Cl⁻ content. K⁺ and Cl⁻ concentrations were varied separately using combinations of KCl, NMDG chloride, potassium gluconate, and NMDG gluconate, for a maximal total concentration of 60 mM; an uptake solution with 50 mM K⁺-Cl⁻ did not contain NMDG chloride, potassium gluconate, or NMDG gluconate, for example. All uptake solutions also contained 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Hepes, pH 7.4, and were supplemented with 1 mM ouabain and 5.0 µCi/ml ⁸⁶Rb⁺. Isotonic conditions were generated by supplementing the same solutions with 3.5 g/100 ml sucrose to reach isosmolar conditions for oocytes (~210 mosmol/kg). Ouabain was added to prevent ⁸⁶Rb⁺ uptake via the Na⁺-K⁺-ATPase. The absence of extracellular Na⁺ and the hypotonicity of the uptake medium prevented ⁸⁶Rb⁺ uptake via the endogenous Na⁺-K⁺-2Cl⁻ cotransporter that is present in oocytes (26).

All uptakes were performed at 32 °C temperature. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without iso-osmole to remove extracellular fluid tracer. Oocytes were dissolved in 10% SDS, and tracer activity was determined for each oocyte by β-scintillation counting.

To determine the ion transport kinetics of KCC4 and KCC1, we performed experiments using varying concentrations of K⁺ and Cl⁻. The sensitivity for several inhibitors was assessed by exposing groups of oocytes to the inhibitors at concentrations varying from 20 µM to 2 mM. For these experiments, the desired concentration of the inhibitor was present during both the incubation and uptake periods, except when noted.

Statistical Analysis.—Statistical significance is defined as two-tailed *p* < 0.05, and the results are presented as mean ± S.E. The significance of the differences between groups was tested by one-way analysis of variance with multiple comparison using Bonferroni correction or by the Kruskal-Wallis one-way analysis of variance on ranks with the Dunn's method for multiple comparison procedures, as needed.

RESULTS

Heterologous Expression of KCC4 and KCC1 in Xenopus Oocytes.—In isotonic conditions, no differences were observed among KCC4, KCC1, and water-injected oocytes (data not shown). When uptakes were performed under hypotonic conditions, microinjection of KCC4 and KCC1 cRNAs resulted in significant K⁺-Cl⁻ cotransport activity, as compared with control oocytes that were injected with water. Fig. 1 summarizes five experiments in which oocytes from different frogs were injected with water or KCC4 or KCC1 cRNA, followed by ⁸⁶Rb⁺ uptake assay using a hypotonic uptake solution containing 10 and 50 mM of extracellular K⁺ and Cl⁻, respectively. In control oocytes, ⁸⁶Rb⁺ uptake was 588 ± 91 pmol/oocyte⁻¹h⁻¹ in the presence of Cl⁻ and 147 ± 23 pmol/oocyte⁻¹h⁻¹ in the absence of Cl⁻, indicating the presence of an endogenous K⁺-Cl⁻ cotransporter. Microinjection of KCC4 cRNA resulted in an increased ⁸⁶Rb⁺ uptake to 24,457 ± 3,476 pmol/oocyte⁻¹h⁻¹. This ⁸⁶Rb⁺ uptake was Cl⁻-dependent, in that uptake in KCC4 oocytes in the absence of extracellular Cl⁻ was 1723 ± 402 pmol/oocyte⁻¹h⁻¹. In oocytes microinjected with KCC1, ⁸⁶Rb⁺ uptake increased to 12,632 ± 2205 pmol/oocyte⁻¹h⁻¹, and the

¹ D. B. Mount and L. Song, unpublished data.

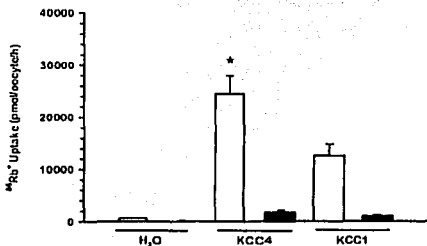


Fig. 1. $^{86}\text{Rb}^+$ Uptake in *X. laevis* oocytes microinjected with water or with cRNA *in vitro* transcribed from KCC4 or KCC1 cDNA as indicated. Uptakes were performed in hypotonic solutions with 120 μM Cl^- in the presence (open bars) or absence (black bars) of extracellular Cl^- . Each bar represents a mean of 90 oocytes extracted from five different frogs. $^{86}\text{Rb}^+$ uptake was performed during 60 min. *, significantly different from uptake in KCC1 control group ($p < 0.01$).

influx was Cl^- -dependent. The difference in the amount of uptake between KCC4 and KCC1 was statistically significant ($p < 0.05$). Although equal amounts of KCC4 and KCC1 cRNA were injected for all experiments that we performed during the study, using multiple cRNA preparations, the relative expression level under hypotonic conditions has always been KCC4 > KCC1. In addition, for each KCC, the absolute uptake varied from frog to frog. Although KCC1 and KCC4 were also compared head-to-head using oocytes from the same frog for individual experiments, to ease the comparison of the two isoforms we present much of the results as the percentage of Cl^- -dependent $^{86}\text{Rb}^+$ uptake. Thus, 100% generally represents the uptake observed in the KCC4 or KCC1 control group minus uptake observed in the water-injected oocytes.

Inhibitor Profile of KCC4 and KCC1—The effect of the loop diuretics furosemide and bumetanide was initially assessed using two different concentrations of extracellular K^+ : 2 and 50 mM. In uptake medium with a K^+ concentration of 2 mM, relative KCC4 activity was 61 ± 3 and $90 \pm 4\%$ in the presence of 2 mM furosemide or bumetanide, respectively. Interestingly, the inhibition of KCC4 by loop diuretics was augmented when the uptake medium contained 50 mM K^+ ; under these conditions, the KCC4 activity was 9 ± 4 or $17 \pm 4\%$ in the presence of furosemide or bumetanide, respectively. In contrast, for KCC1 this effect of extracellular K^+ was not observed for furosemide and was marginal for bumetanide. KCC1 function in the presence of furosemide was $9 \pm 2\%$ in 2 mM K^+ and $18 \pm 8\%$ in 50 mM K^+ (p not significant), and in the presence of bumetanide it was 51 ± 12 versus $19 \pm 7\%$ in 2 and 50 mM K^+ , respectively ($p = 0.05$; $t = 1.99$). To further define the differences in the K^+ effect on the sensitivity to loop diuretics between KCC4 and KCC1, we assessed the inhibitory effect of furosemide and bumetanide at several concentrations of extracellular K^+ . The results of these series of experiments are shown in Fig. 2. The percentage inhibition of KCC4 by both furosemide and bumetanide was significantly affected by extracellular K^+ (Fig. 2, upper panels). The minimal and maximal inhibition by both loop diuretics was observed at 2 and 6 mM, respectively; no further effect was observed at higher K^+ concentrations. In contrast, the percentage of KCC1 inhibition by either furosemide or bumetanide did not vary as a function of extracellular K^+ concentration (Fig. 2, lower panels). Thus, to define differences between the two KCCs in sensitivity to loop diuretics, we used a 10 mM concentration of extracellular

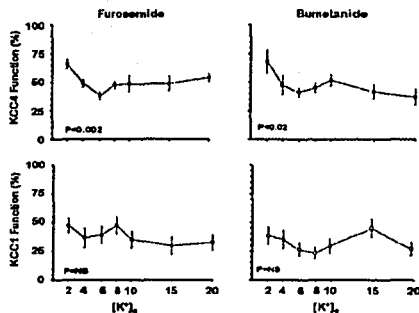


Fig. 2. Effect of extracellular K^+ concentration ($[\text{K}^+]$) on the inhibition of $^{86}\text{Rb}^+$ uptake by the loop diuretics furosemide and bumetanide. Data from KCC4-injected oocytes are presented in the upper panels, and data from KCC1-injected oocytes are shown in the lower panels. In all experiments, the Cl^- concentration of the extracellular medium was 60 mM, whereas the $[\text{K}^+]$ increased from 2 to 20 mM. The mean uptake in the absence of loop diuretic for each KCC was taken as the 100% of uptake, and data from diuretic-treated groups was normalized to the uptake in this control group. Experimental groups were exposed to a 2 mM concentration of furosemide or bumetanide during the incubation and uptake periods. Each point represents the mean \pm S.E. of at least 15 oocytes.

K^+ to assess the concentration curves for furosemide and bumetanide inhibition upon the Cl^- -dependent $^{86}\text{Rb}^+$ uptake in KCC4 or KCC1. As Fig. 3 illustrates, KCC4 exhibits apparent half-maximal inhibition ($K_{0.5}$) values of $\sim 900 \mu\text{M}$ for both furosemide and bumetanide. These are lower than the respective values for KCC1 ($\sim 180 \mu\text{M}$ for furosemide and bumetanide). Therefore, KCC4 clearly exhibits a lower affinity for loop diuretics than does KCC1. The inhibition of KCC1 by furosemide in Fig. 3 suggests the possibility of a second affinity site for the loop diuretic. However, this inhibition fitted well to a Michaelis-Menten kinetics pattern with one inhibitor-binding site. The data did not fit to an equation with two binding sites (data not shown).

The sensitivity of the KCCs to other inhibitors of red cell K^+ - Cl^- cotransport was also assessed in oocytes injected with KCC4 or KCC1. Fig. 4 illustrates the effect of 100 μM DIDS and 100 μM DIOA on the $^{86}\text{Rb}^+$ uptake induced by the microinjection of each KCC cRNA. The effect of extracellular K^+ concentration on the inhibition of cotransport was very dramatic for DIDS. When the concentration of extracellular K^+ was 2 mM, the addition of DIDS to the extracellular medium resulted in reduction of KCC4 function to $65 \pm 10\%$ ($p < 0.003$) and of KCC1 to $85 \pm 6\%$ ($p = 0.113$, not significant). In contrast, when 50 mM of extracellular K^+ was used, DIDS resulted in significant decrease of KCC4 and KCC1 to 13 ± 4 and $12 \pm 2\%$, respectively. The addition of 100 μM of DIOA to the extracellular medium also resulted in inhibition of the KCCs. However, inhibition of KCC4 was higher when extracellular K^+ was lower, although this was not the case for KCC1. DIOA is reportedly specific for K^+ - Cl^- cotransport over Na^+ - K^+ - 2Cl^- cotransport (27), and the same concentration of DIOA had no effect on the function of the Na^+ - K^+ - 2Cl^- cotransport activity of *Xenopus* oocytes (26) (data not shown).

We also tested the effect of a 2 mM concentration of the thiazide diuretic trichlormethiazide on the percentage of chlo-

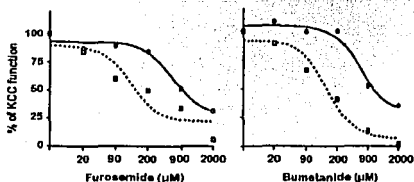


Fig. 3. Concentration-response profiles for inhibition of KCC4 (circles) and KCC1 (squares) by furosemide (left panel) or bumetanide (right panel). Groups of 16 *Xenopus* oocytes microinjected with KCC4 or KCC1 were exposed to increased concentrations of furosemide or bumetanide in the preincubation and uptake medium, from 20 to 2000 μ M. Data were normalized as the percentage of influx in each KCC, taking 100% as the value observed in oocytes in which uptake was done in the absence of loop diuretics. Each point represents the mean \pm S.E. of at least 15 oocytes.

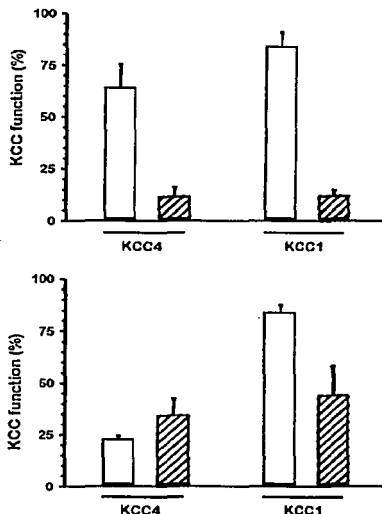


Fig. 4. Effect of the inhibitors DIDS (upper panel) and DIOA (lower panel) upon ⁸⁶Rb⁺ uptake in KCC4- and KCC1-injected oocytes incubated in hypotonic conditions (120 mosmol/kg), in the presence of an extracellular K⁺ concentration of 2 mM (open bars) or 50 mM (hatched bars). The Cl⁻ concentration of the extracellular medium was 50 mM in both conditions. In all experiments, ⁸⁶Rb⁺ uptake was assessed in control groups of 2 and 50 mM K⁺ concentration, in the absence of inhibitors, and experimental groups were exposed to a 100 μ M of DIDS or DIOA during incubation and uptake periods. Each bar represents the mean \pm S.E. of at least 15 oocytes.

side-dependent ⁸⁶Rb⁺ uptake. Surprisingly, given the supposed specificity of thiazides for Na⁺Cl⁻ cotransport (28), KCC4 was moderately sensitive to trichlormethiazide. As we observed with furosemide and DIDS, the higher the extracellular K⁺,

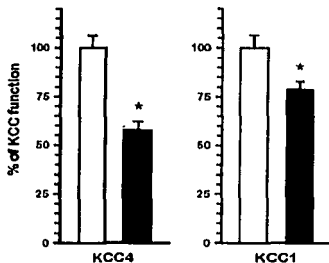


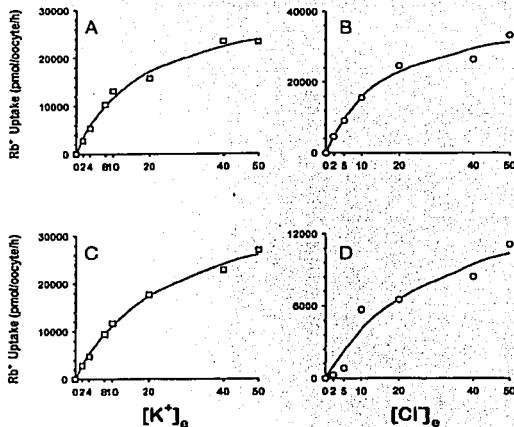
Fig. 5. Effect of 10 mM BaCl₂ upon the ⁸⁶Rb⁺ influx induced by microinjections of oocytes with KCC4 or KCC1 cRNA. Uptakes in the control groups were performed using a hypotonic uptake medium containing 40 mM NMDG chloride and 10 mM KCl, and uptakes in the BaCl₂ group were performed using a hypotonic medium containing 30 mM NMDG chloride, 10 mM BaCl₂, and 10 mM KCl. Each bar represents a mean of 20 oocytes. Open bars represent the normalized influx in control group, and black bars show normalized influx in BaCl₂ groups. *, $p < 0.01$ versus uptake in control group.

the higher the inhibition by thiazides, since in 2 mM of extracellular K⁺ ⁸⁶Rb⁺ uptake was reduced to 79 \pm 3%, and at 60 mM it was reduced to 57 \pm 9%. This difference was significant ($p < 0.01$). In KCC1-injected oocytes, trichlormethiazide reduced ⁸⁶Rb⁺ uptake by a statistically significant amount to 64 \pm 4% in 2 mM K⁺; this inhibitory effect was not statistically significant at 50 mM K⁺ (74 \pm 8% reduction in activity). Consistent sensitivity to trichlormethiazide is thus unique to KCC4.

Independent studies have suggested that barium can inhibit renal K⁺Cl⁻ cotransporters (7, 8, 29). We thus assessed the effect of 10 mM extracellular barium on the function of KCC4 and KCC1. Fig. 5 shows that when 10 mM BaCl₂ was added to the uptake medium, KCC4-induced influx was reduced to 58 \pm 4.3% of the uptake observed in KCC4-injected control oocytes. KCC1 function was only reduced to 79 \pm 4.2%, hence the inhibitory effect of barium was significantly greater for KCC4 than for KCC1 ($p < 0.01$).

Kinetic Properties of KCC4 and KCC1—To determine and compare the kinetic properties of KCC4 and KCC1 in the same expression system, we measured ⁸⁶Rb⁺ uptake in KCC4- and KCC1-injected oocytes as a function of the concentration of each transported ion. The results of these series of experiments are depicted in Fig. 6. Uptakes were performed with K⁺ or Cl⁻ fixed at 50 mM, with changing concentrations of the counterion from 0 to 50 mM. Uptakes were also measured in water injected oocytes (data not shown), and the mean values for water injected oocytes were subtracted from corresponding KCC groups in order to assess only the ⁸⁶Rb⁺ uptake mediated by each heterologously expressed isoform. As shown in Fig. 1, ⁸⁶Rb⁺ uptake in water-injected oocytes was low, such that this correction was generally minor. In the case of KCC4, ⁸⁶Rb⁺ influx increased as the concentration of each transported ion was raised, until a plateau phase was reached at ion concentrations greater than 20–40 mM, compatible with Michaelis-Menten behavior. The calculated apparent K_m and V_{max} for extracellular K⁺ concentration were 17.5 \pm 2.7 mM and 32,370 \pm 2115 pmol/oocyte⁻¹h⁻¹, respectively. The calculated apparent K_m and V_{max} values for extracellular Cl⁻ concentration were 16.12 \pm 4.2 mM and 41,440 \pm 4174 pmol/oocyte⁻¹h⁻¹, respectively. The Hill coefficient for both ions remained close to unity;

Fig. 6. Kinetic analysis of $^{86}\text{Rb}^+$ uptake using hypotonic conditions in oocytes injected with cRNA from KCC4 (A and B) or KCC1 (C and D). A and C, K^+ dependence of $^{86}\text{Rb}^+$ uptake for each KCC. B and D, Cl^- dependence of $^{86}\text{Rb}^+$ uptake for each KCC. Uptakes were performed with K^+ or Cl^- fixed at 50 mM, varying the concentration of the appropriate counterion from 0 to 50 mM, as indicated. Uptakes were also measured in water-injected oocytes (data not shown), and the mean values for the corresponding water groups were subtracted to analyze only the $^{86}\text{Rb}^+$ uptake due to each injected KCC. Lines were fit using the Michaelis-Menten equation. Data are expressed as uptakes in pmol/oocyte $^{-1}\text{h}^{-1}$; each point represents the mean of at least 25 oocytes.



1.08 ± 0.2 and 1.06 ± 0.3 for K^+ and Cl^- , respectively. KCC1 also exhibited a similar Michaelis-Menten behavior. The apparent K_m and V_{max} in KCC1 were 25.5 ± 3.2 mM and $39,540 \pm 2199$ pmol/oocyte $^{-1}\text{h}^{-1}$ for extracellular K^+ and 17.2 ± 8.3 mM and $14,930 \pm 2822$ pmol/oocyte $^{-1}\text{h}^{-1}$ for Cl^- . Hill coefficients for K^+ (1.04 ± 0.13) and Cl^- (1.3 ± 0.5) in KCC1 also were close to unity.

Anion Dependence of KCC4 and KCC1—It has been shown that some extracellular anions other than Cl^- can support ion translocation through the $\text{K}^+\text{-Cl}^-$ cotransporter of both sheep and human erythrocytes (30). It was thus of interest to measure $^{86}\text{Rb}^+$ transport by KCC4 and KCC1 in the presence of different anions. The $^{86}\text{Rb}^+$ influx of KCC4- and KCC1-injected oocytes using an uptake solution containing 40 mM potassium gluconate and 10 mM KCl served as the reference activity for these experiments, as compared with uptake activity in oocytes exposed to medium containing 40 mM potassium gluconate and 10 mM of KBr, KH_2PO_4 , KI, potassium gluconate, or KSCN. Fig. 7 shows the percentage of KCC4 (upper panel) and KCC1 (lower panel) function when uptakes were performed using these different anion substitutions. KCC4 shows the higher $^{86}\text{Rb}^+$ influx in the presence of 10 mM KCl. $^{86}\text{Rb}^+$ influx was still observed in the presence of other anions: $58 \pm 9\%$ with 10 mM KBr, $22 \pm 5.9\%$ with 10 mM KH_2PO_4 , and $17 \pm 3.8\%$ with KI, whereas potassium gluconate and KSCN did not support transport. These results are in contrast to those observed in KCC1-injected oocytes, for which the order of anion-supported transport was $\text{Cl}^- > \text{SCN}^- = \text{Br}^- > \text{PO}_4^{3-} > \text{I}^- > \text{gluconate}$.

Regulation of KCC4 and KCC1—One of the most distinctive characteristics of $\text{K}^+\text{-Cl}^-$ cotransport in several cells and species is activation by the alkylating agent NEM (2). We therefore analyzed the effect of NEM on $^{86}\text{Rb}^+$ influx in groups of oocytes under isotonic or hypotonic conditions. Again, in all of the experiments in which we assessed $^{86}\text{Rb}^+$ influx in oocytes that were incubated in isotonic medium (~ 210 mosmol/kg), the uptake observed in KCC4- or KCC1-injected oocytes was not different from the uptake in water-injected oocytes. However, the addition of 1 mM NEM in isotonic conditions resulted in a

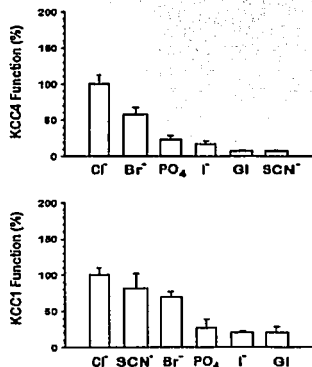
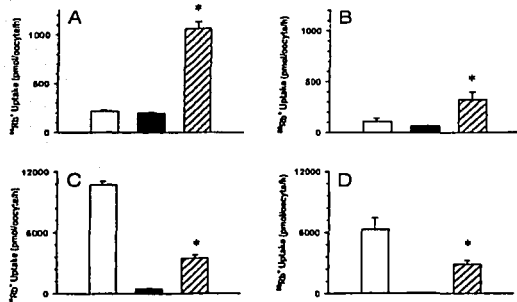


Fig. 7. Anion dependence of KCC4 (upper panel) and KCC1 (lower panel). $^{86}\text{Rb}^+$ influx was assessed in hypotonic uptake medium containing 40 mM NMDG gluconate plus 10 mM concentration of KCl (control group) or 10 mM potassium salts of each of the anion substitutes (KBr, KH_2PO_4 , KI, potassium gluconate, and KSCN). Preincubation was done in a solution containing 50 mM NMDG gluconate. Data were normalized taking uptake in the KCl group as 100%. Each bar represents the mean \pm S.E. of at least 15 oocytes.

5-fold activation of KCC4 (214 ± 12 pmol/oocyte $^{-1}\text{h}^{-1}$ in the KCC4 control group versus 1062 ± 70 pmol/oocyte $^{-1}\text{h}^{-1}$ in the NEM-treated group, $p < 0.001$) and a 2.6-fold activation of KCC1 (120 ± 27 versus 319 ± 76 pmol/oocyte $^{-1}\text{h}^{-1}$, $p < 0.05$) (Fig. 8, A and B). Of note, when uptakes were performed in hypotonic medium, the addition of NEM resulted in a dramatic inhibition of both isoforms (Fig. 8, C and D), such that $^{86}\text{Rb}^+$

FIG. 8. Effect of 1 mM NEM on the $^{86}\text{Rb}^+$ uptake in KCC4-injected (A and C) and KCC1-injected (B and D) oocytes, under both isotonic (A and B) and hypotonic (C and D) conditions. Each bar represents the mean of 20 oocytes. Uptakes in the control conditions are shown in open bars, in the absence of extracellular Cl^- in black bars, and in the presence of NEM in hatched bars. *, $p < 0.0001$ versus control group.



uptakes induced by KCC4 and KCC1 were reduced by 68 and 55%, respectively. In the same experiments, $^{86}\text{Rb}^+$ uptake due to the endogenous oocyte K^+/Cl^- cotransporter (H_2O -injected oocytes) was significantly increased when uptakes were done under both isotonic and hypotonic conditions (data not shown).

It has been known for some time that the inhibition of protein phosphatases prevents the activation of red cell K^+/Cl^- cotransport by either cell swelling or NEM. Since the role of phosphatases in the control of the cloned KCCs is unclear, we studied the effect of three inhibitors of protein phosphatases. We used 100 nM calyculin A, which inhibits the function of protein phosphatases 1 and 2A. The relative role of specific phosphatases was assessed using okadaic acid at 1 nM, a concentration that only affects protein phosphatase 2A, and cypermethrin at 100 pM, a concentration in which this compound inhibits the function of protein phosphatase 2B. As Fig. 9 shows, the addition of calyculin A completely prevents the activation of KCC4 and KCC1 by cell swelling. In contrast, neither okadaic acid, nor cypermethrin prevented this activation. These results indicate that protein phosphatase 1 is required for the activation of both KCC4 and KCC1 by cell swelling.

DISCUSSION

We have recently shown (16) that heterologous expression of the mouse KCC4 cDNA induced the expression of a $^{86}\text{Rb}^+$ influx pathway that is activated by cell swelling, dependent on the presence of extracellular Cl^- , and inhibited by 2 mM of the loop diuretic furosemide. These data established that KCC4 functions as a K^+/Cl^- cotransporter. The present study extends these initial observations and defines the functional properties of KCC4 in greater detail. In addition, we have studied the functional properties of KCC1, the other widely distributed KCC isoform, using the same experimental protocols. Since sequence comparisons of the four KCC proteins pair KCC4 with KCC2 and KCC1 with KCC3, this study also constitutes the first direct functional comparison of the two subgroups of the KCCs.

Our data indicate that KCC1 and KCC4 express minimal K^+/Cl^- cotransport in unstimulated cells under isotonic conditions but are strongly activated by cell swelling induced by hypotonic conditions. In our expression system, neither KCC4 nor KCC1-injected oocytes exhibited a significant increase of $^{86}\text{Rb}^+$ uptake over water-injected oocytes when incubated in isotonic media during the influx period. When oocytes were incubated in hypotonic conditions, however, both cotransport-

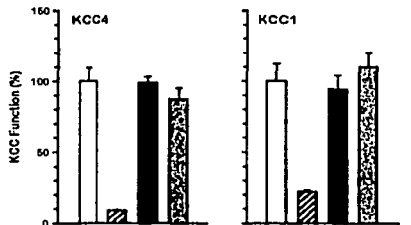


FIG. 9. Effect of the protein phosphatase inhibitors calyculin A (100 nM) (hatched bar), okadaic acid (1 nM) (black bar), and cypermethrin (100 pM) (gray bar) upon the swelling-induced activation of KCC4 or KCC1. In both panels, the white bar represents the control group. $^{86}\text{Rb}^+$ influx in hypotonic medium in the absence of inhibitor. Each bar represents the mean \pm S.E. of at least 15 oocytes.

ers were markedly activated, albeit with a different magnitude (KCC4 > KCC1). These findings differ from previous observations (13, 15, 17, 19, 20) that indicate minimal hypotonic activation of KCC1, KCC2, and KCC3 when these cotransporters were expressed in HEK 293 cells, but agree with the cell swelling-induced activation of KCC1 found by Su *et al.* (18) using *Xenopus* oocytes as an expression system. Thus, when expressed in *Xenopus* oocytes, KCC4 and KCC1 cotransporters can be activated by cell swelling, suggesting that HEK 293 cells may not possess the appropriate signaling pathways for the activation of the cotransporters by swelling. In comparison, volume-regulated transport pathways recapitulate their *in vivo* physiology when expressed in *Xenopus* oocytes. Thus, shrinkage-activated transport pathways such as the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (26) or the epithelial sodium channel ENaC (31) and swelling-activated pathways such as the calcium-activated intermediate K^+ channel $\text{mI}K_1$ (32) are regulated appropriately in this expression system. Of the four KCCs, KCC4 seems to be the isoform that exhibits the highest activation by hypotonicity. Influx mediated by KCC4 in this study was consistently higher than KCC1, although the amount of injected cDNA was equivalent. There are, however, several possible explanations for the greater hypotonic activation of KCC4. For example, KCC4 cRNA may be intrinsically more stable or better translated than KCC1 in oocytes. Of note, however, rat

KCC2 reportedly encodes a K⁺-Cl⁻ cotransporter with significant activity under isotonic conditions when expressed in *Xenopus* oocytes and only minimal activation by cell swelling (21). Therefore, it is likely that much of the observed differences in volume sensitivity is due to variation in the structure of the four KCC proteins.

The two major loop diuretics inhibit KCC4, with an inhibitor sensitivity that is lower than that observed for KCC1. The reported effect of external potassium ([K⁺]_o) on the inhibition of K⁺-Cl⁻ cotransport by loop diuretics (33) was observed for KCC4 but not KCC1. We observed a significantly different effect of furosemide and bumetanide on KCC4 at variable [K⁺]_o, with the minimal and maximal effect at [K⁺]_o of 2 and 6 mM, respectively. In contrast, no effect of [K⁺]_o was observed for the inhibition of KCC1 by loop diuretics. This finding suggests that, as the isoform with the lower inhibitor affinity, the inhibition of KCC4 is more dependent on the positive effect of [K⁺]_o on the interaction between the transporter protein and loop diuretics. Similar to our loop diuretic experiments, the anion transport inhibitor DIDS inhibited the function of KCC4 and KCC1, with an apparent K_i that was dramatically lowered by an increase in [K⁺]_o. Almost no effect was observed at 2 mM [K⁺]_o, while in 50 mM [K⁺]_o, ⁸⁶Rb⁺ influx was completely blocked by a 100 μM DIDS concentration. This relationship between [K⁺]_o and the inhibition of the K⁺-Cl⁻ cotransporter by DIDS was previously observed in low potassium sheep red blood cells (34) and was explained by the existence of two sites for K⁺ in the cotransporter: a modifier site and a transport site. Of interest, DIDS can also inhibit the function of the thiazide-sensitive Na⁺-Cl⁻ cotransporter but has no effect on the bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter (26). The acid alkaloid DIOA, considered a specific inhibitor of red cell K⁺-Cl⁻ cotransport (27), also inhibited KCC4 and KCC1. However, in contrast to DIDS and loop diuretics, all of which primarily inhibit anion transporters and exchangers (35, 36), the higher the [K⁺]_o, the lower the efficacy of DIOA. However, even in a very high [K⁺]_o (50 mM), the inhibition of ⁸⁶Rb⁺ influx by 100 μM DIOA was still greater than 50%.

The increased ⁸⁶Rb⁺ uptake induced by KCC4 was also inhibited by about 20% in low and 40% in high [K⁺]_o, by 2 mM concentration of the thiazide-diuretic trichloromethiazide. The members of the electroneutral cation chloride coupled cotransporters have been defined in part due to their sensitivity to diuretics. The Na⁺-K⁺-2Cl⁻ cotransporters are sensitive to loop diuretics, derivatives of sulfamoylbenzoic acid, and resistant to the benzothiadiazine derivatives, whereas the Na⁺-Cl⁻ cotransporter is inhibited by thiazides but not affected by loop diuretics (12). Our results suggest that KCC4, which exhibits a low degree of identity with the sodium-dependent cation-chloride cotransporters (~22%), can be inhibited not only by loop diuretics but also by thiazide-type diuretics. A similar observation has been reported by Harling *et al.* (37), who showed that the plant cation-chloride cotransporter AXI 4, which exhibits the highest sequence identity with the KCCs (36–38%), can also be inhibited by bumetanide, furosemide, and the thiazide-like diuretic metolazone.

We have found that KCC4 and KCC1 can be blocked by the addition of 10 mM BaCl₂ to the uptake medium, with a relative sensitivity of KCC4 > KCC1. Although red cell K⁺-Cl⁻ cotransport is at least partially sensitive to quinidine derivatives (38), this is the first indication that the cloned K⁺-Cl⁻ cotransporters are directly sensitive to BaCl₂. This observation is also consistent with the controversial proposal by Greger and Schlatter (7) that the basolateral membrane of renal thick ascending limb cells contains a barium-sensitive K⁺-Cl⁻ cotransporter. The observation by Amlal *et al.* (8) that a thick

limb chloride-dependent, barium-sensitive NH₄⁺ transport mechanism is only modestly sensitive to furosemide suggests that KCC4 is the isoform present in the basolateral membrane of the mammalian thick ascending limb. In this regard, Liapis *et al.* (39) failed to detect KCC1 mRNA in human thick ascending limb.

Kinetic analyses reveal that KCC4 and KCC1 exhibit very similar affinities for extracellular Cl⁻ (K_m values in 16.1 ± 4.2 and 17.2 ± 8.3 mM, respectively), whereas the differences in the affinity for extracellular K⁺ approached statistical significance (K_m values 17.5 ± 2.7 and 25.5 ± 3.2 mM, respectively; *p* = 0.08). Our results for KCC1 agree with those reported by Gillen *et al.* (13). It is known that the central core of 12 transmembrane (TM) segments determines the kinetic properties of the cation-chloride cotransporters (40). In this regard, the otherwise identical TM segments of the four KCC proteins differ primarily at amino acid residues within TM2, TM4, and TM7. An elegant series of studies have implicated TM2 in the determination of cation (Na⁺ and K⁺) affinity in the Na⁺-K⁺-2Cl⁻ cotransporters, whereas residues within TM4 and TM7 appear to affect anion affinity (23, 24, 40, 41). However, sequence comparisons of the entire gene family indicate that only TM7 is particularly conserved between the Na⁺-dependent and Na⁺-independent cation-chloride cotransporters; thus, these observations may not translate to the K⁺-Cl⁻ cotransporters. Of particular significance, however, the reported affinity of KCC2 for K⁺ (~5 mM) is closer to that of KCC4 than KCC1, and KCC2 shares significant identity with KCC4 within TM2. Therefore, as in the Na⁺-K⁺-2Cl⁻ cotransporters, TM2 may play a major role in the determination of cation affinity. The K⁺ affinity of individual K⁺-Cl⁻ cotransporters may be of major physiological significance, since under conditions wherein extracellular K⁺ increases, such as cardiac ischemia (9) and neuronal activity (4), the higher affinity KCCs may function as K⁺-Cl⁻ influx pathways.

KCC4 and KCC1 exhibit surprisingly similar affinities for chloride. However, they do differ in another parameter of anion transport, the anion selectivity or "anion-series" of K⁺-Cl⁻ cotransport. KCC4 and KCC1 thus differ in the profile of ⁸⁶Rb⁺ transport that can be sustained by different anions. In KCC4, about 50% of the function can be observed in the presence of Br⁻, and some transport is still present with PO₄³⁻, whereas in KCC1 70% of ⁸⁶Rb⁺ uptake can be obtained in the absence of Cl⁻, when either Br⁻ or SCN⁻ are present in the extracellular medium. Whether these differences are encoded by subtle variation in TM4 and TM7 will require further study; however, differences in anion selectivity were crucial for the identification of the anion channel pore in the CLC chloride channels (42, 43).

The functional properties of KCC4 and KCC1 observed in the present study suggest that it is unlikely that either of these isoforms is the predominant K⁺-Cl⁻ cotransporter expressed in red blood cells. On the one hand, Delpire and Lauf (44) observed that the K⁺-Cl⁻ cotransporter in hypototically swollen low K sheep erythrocytes exhibited a K_m for extracellular K⁺ of ~55 mM, which is very different from the K_m obtained in the present study for KCC4 and KCC1. It has also been shown in sheep red blood cells (45) that K⁺ influx is higher in the presence of Br⁻ than in the presence of Cl⁻; neither KCC4 nor KCC1 exhibited this behavior. Moreover, although it has been shown that KCC1 mRNA is expressed in mouse erythroleukemic cells, it is not present in circulating reticulocytes (46), and the KCC2 isoform is expressed exclusively in the central nervous system (14). Thus, taking all the information together, our kinetic and anion substitution experiments suggest that the major K⁺-Cl⁻ cotransporter in erythrocytes is either KCC3 or

an as-yet-unidentified isoform.

K⁺-Cl⁻ cotransport was initially defined as a red cell transport pathway that is activated by the alkylating agent NEM. Multiple laboratories have since found that pretreatment of erythrocytes from several species with 1 mM NEM results in significant activation of this Cl⁻-dependent K⁺ transport pathway (2). It is still unclear if the activating effect of NEM is related to NEM-induced dephosphorylation, via activation of an upstream kinase, or to direct modification of the thiol groups on the cotransporter. There are reports supporting both possibilities (47) (for reviews, see Refs. 2 and 3). However, like the activation by cell swelling, NEM-activated transport is prevented by phosphatase inhibitors, suggesting a positive effect of NEM on upstream signaling pathways (48–52). In this regard, our data show a very interesting behavior of the KCCs when exposed to NEM. Under isotonic conditions, NEM stimulated the function of KCC4 and KCC1, as well as the endogenous K⁺-Cl⁻ cotransporter of the oocytes. Su *et al.* (18) also observed KCC1 activation by NEM in oocytes. In contrast, in hypotonic conditions, when we exposed oocytes to NEM, both KCC4 and KCC1 were inhibited. The fact that KCC4 and KCC1 were activated by NEM in isotonic conditions suggests that oocytes possess the intracellular pathways that NEM requires for activation of the cotransporters. The mechanisms by which swelling-activated KCC4 and KCC1 are inhibited by NEM are still unclear. It has been shown in sheep red cells that K⁺-Cl⁻ cotransporters can be activated or inhibited by NEM through high and low affinity stimulatory thiols, respectively (47, 53, 54). Further experiments will be required to clarify this issue; however, a reconciliation of these and previous observations is that there are direct inhibitory sites on the transporter proteins themselves and stimulatory sites on upstream kinases. In this regard, there are several transmembrane or juxtamembrane cysteines in the predicted KCC proteins, and transmembrane cysteines were recently implicated in the differential sensitivity of Na⁺-K⁺-2Cl⁻ cotransporters to cysteine-reactive compounds (55).

Over the last decade or so, several laboratories have suggested that dephosphorylation of the K⁺-Cl⁻ cotransporter is required for its activation, since inhibition of protein phosphatases prevents the swelling- and NEM-induced activation (48–50, 56, 57). Our data support this hypothesis, since the protein phosphatase inhibitor calyculin A completely abrogates hypotonic activation of KCC4 and KCC1. Calyculin A is known to inhibit both protein phosphatase 1 and 2A (58). To discriminate between phosphatases, we also tested the effect of okadaic acid in a concentration of 1 nM, which inhibits only protein phosphatase 2A, and cypermethrin, which inhibits only protein phosphatase 2B (59, 60). Since these two compounds did not affect activation of KCCs, we suggest that, at least in *Xenopus* oocytes, protein phosphatase 1 is the phosphatase that is involved in activation of the KCCs during cell swelling. In addition to a role in cell volume regulation, activation of K⁺-Cl⁻ cotransport by protein phosphatase 1 may play a role in trans-epithelial transport of salt in the kidney and other epithelial organs. There is evidence for a swelling-activated basolateral K⁺-Cl⁻ cotransporter in the proximal tubule (61, 62), where protein phosphatase 1 also is responsible for activating the basolateral Na⁺-K⁺-ATPase (63); protein phosphatase 1 may thus function to couple the pump to basolateral Cl⁻ and K⁺ exit through the KCCs. Of more specific relevance to K⁺-Cl⁻ cotransport, the combined data for NEM, phosphatase inhibition, and cell swelling dissociates for the first time these various control points for this transport pathway.

In conclusion, we have found significant regulatory, kinetic, and pharmacological differences between KCC4 and KCC1.

Despite differences in their relative activation by cell swelling, KCC4 and KCC1 share a requirement for dephosphorylation by a protein phosphatase for swelling-induced activity. As previously shown for K⁺-Cl⁻ cotransport in red cells (48, 50, 56, 64), the relevant protein phosphatase is probably protein phosphatase 1. The two K⁺-Cl⁻ isoforms in this study differ slightly in affinity for K⁺, presumably due to variation within transmembrane 2, a region of the cation-chloride cotransporter proteins previously implicated in cation affinity (22–24). Ion affinity may also be of physiological relevance, in that lower affinity K⁺-Cl⁻ cotransporters, such as KCC1 and the red cell K⁺-Cl⁻ cotransporter (potentially KCC3), may function exclusively as efflux mechanisms. As proposed initially by Payne (19), the higher affinity isoforms (KCC2 and now KCC4) may function as both efflux and influx pathways. Such a duality has been verified experimentally in neurons (65), where synaptic activity may increase extracellular K⁺ to the point that KCC2 mediates K⁺-Cl⁻ influx. KCC4 transcript is in turn particularly abundant in heart, where K⁺ efflux during ischemia appears to involve K⁺-Cl⁻ cotransport (9); again, a higher affinity isoform may play a role in reclaiming this intracellular K⁺ in the postischemic myocardium. Finally, the pharmacological characterization of KCC4 fulfills the prediction, based on observations of the physiology of renal thick ascending limb cells (7, 66), that K⁺-Cl⁻ cotransporters may be sensitive to barium, widely considered a specific inhibitor of K⁺ channels.

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Functional and molecular characterization of the K-Cl cotransporter of *Xenopus laevis* oocytes

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Mercado, Adriana, Paola de los Heros, Norma Vázquez, Patricia Meade, David B. Mount, and Gerardo Gamba. Functional and molecular characterization of the K-Cl cotransporter of *Xenopus laevis* oocytes. *Am J Physiol Cell Physiol* 281: C670–C680, 2001.—The K-Cl cotransporters (KCCs) have a broad range of physiological roles, in a number of cells and species. We report here that *Xenopus laevis* oocytes express a K-Cl cotransporter with significant functional and molecular similarity to mammalian KCCs. Under isotonic conditions, defolliculated oocytes exhibit a Cl⁻-dependent ⁸⁶Rb⁺ uptake mechanism after activation by the cysteine-reactive compounds *N*-ethylmaleimide (NEM) and mercuric chloride (HgCl₂). The activation of this K-Cl cotransporter by cell swelling is prevented by inhibition of protein phosphatase-1 with calyculin A; NEM activation of the transporter was not blocked by phosphatase inhibition. Kinetic characterization reveals apparent values for the Michaelis-Menten constant of 27.7 ± 3.0 and 15.4 ± 4.7 mM for Rb⁺ and Cl⁻, respectively, with an anion selectivity for K⁺ transport of Cl⁻ = PO₄³⁻ = Br⁻ > I⁻ > SCN⁻ > gluconate. The oocyte K-Cl cotransporter was sensitive to several inhibitors, including loop diuretics, with apparent half-maximal inhibition values of 200 and 500 μM for furosemide and bumetanide, respectively. A partial cDNA encoding the *Xenopus* K-Cl cotransporter was cloned from oocyte RNA; the corresponding transcript is widely expressed in *Xenopus* tissues. The predicted COOH-terminal protein fragment exhibited particular homology to the KCC1/KCC3 subgroup of the mammalian KCCs, and the functional characteristics are the most similar to those of KCC1 (Mercado A, Song L, Vázquez N, Mount DB, and Gamba G. *J Biol Chem* 276: 30326–30334, 2000).

potassium-chloride cotransport; cell volume; cell swelling

THE ELECTRONEUTRAL COTRANSPORT OF K⁺ AND Cl⁻ is largely accomplished by parallel K⁺ and Cl⁻ channels or via the operation of K-Cl cotransporters. K-Cl cotransport was first defined in the red blood cell (10, 32), the tissue for which functional characterization is the most complete. Red blood cell K-Cl cotransport shares a number of functional properties with Na-K-2Cl cotransport, including electroneutral characteristics, a functional

dependence on the presence of each transported ion, and sensitivity to loop diuretics. However, these two transport mechanisms diverge significantly in several characteristics, in particular their response to cellular volume changes and to modulation of protein phosphorylation and dephosphorylation. The Na-K-2Cl cotransport is thus shrinkage activated and inhibited by protein phosphatases (45), whereas K-Cl cotransport is activated by cell swelling and completely abolished by inhibitors of serine/threonine protein phosphatases (10).

In addition to red blood cells, K-Cl cotransport has been detected in a variety of different tissues and cells, including neurons (48), epithelia (3, 17), myocardium (59), skeletal muscle (58), and vascular smooth muscle (2). Four mammalian K-Cl cotransporter isoforms were recently cloned and designated KCC1 (16), KCC2 (44), KCC3 (20, 40), and KCC4 (40).¹ K-Cl cotransport activity has also been demonstrated in several nonmammalian cells, including teleost erythrocytes and hepatocytes (5, 8, 18, 26), amphibian red blood cells (19), lobster neurons (56), and malpighian tubules from both *Drosophila melanogaster* (34) and the forest ant *Formica polyctena* (33). The physiological roles of K-Cl cotransport remain poorly understood. However, activation by cell swelling suggests a prominent role for KCCs in the regulatory volume decrease of cells exposed to hypotonic conditions or swollen by cellular insults such as ischemia. There is also evolving evidence for the participation of K-Cl cotransport in trans-epithelial salt transport and intracellular ion homeostasis (3, 17, 48, 56).

¹ We initially referred to the KCC on human chromosome 15q14 as KCC4 and the KCC on chromosome 5p15 as KCC3 (40). However, in deference to the earlier publication of Hiki et al (20), we reversed the numbering of our GenBank/EBI submissions to refer to the KCC on chromosome 15q14 as KCC3 and the KCC on chromosome 5p15 as KCC4 (SEE NOTE ADDED IN PROOF IN Ref. 40).

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During the course of the cloning and characterization of electroneutral cation-chloride cotransporters (13, 14, 38–40, 46), we initially observed that oocytes from the frog *Xenopus laevis* do not contain thiazide-sensitive Na-Cl cotransport (14, 39) but do express an endogenous bumetanide-sensitive Na-K-2Cl cotransporter that can be activated by hypertonic conditions (13) and inhibited by activation of protein kinase C (47). Suvitayavat et al. (54) and Shtelar et al. (51) have reported similar findings. More recently we and others have obtained preliminary evidence that *Xenopus* oocytes also contain an endogenous K-Cl cotransporter (38, 40, 53). During reproduction frogs place their oocytes in hypotonic pond water, resulting in profound cellular swelling in the absence of a compensatory response. Although swelling-activated K-Cl cotransport has not been reported in oocytes, these cells are known to possess hypotonically activated Cl⁻ channels (1). Once the oocytes become fertilized eggs, they are particularly resistant to cell swelling, potentially because of conformational changes of the cytoskeleton that reduce the capacity of the cell to swell (28). A physiological response to hypotonicity has also been demonstrated in *X. laevis* spermatozoa, which remain immotile until the osmolarity of the semen is diluted in pond water (21).

To begin to study the role of K-Cl cotransport in *X. laevis*, we initiated a molecular and functional study of the oocyte transporter. In addition, since *Xenopus* oocytes are used for the characterization of other KCCs, functional characterization was necessary to understand the regulation of the endogenous transporter and to minimize the misinterpretation of heterologous expression. We report here that *Xenopus* oocytes exhibit an endogenous K-Cl cotransporter that can be activated by hypotonicity. We also describe the major kinetic parameters of the cotransporter, as well as its inhibitory and regulatory profile. *Xenopus* oocytes also express mRNA encoding a K-Cl cotransporter protein with significant homology to the mammalian KCCs. This constitutes the first detailed functional characterization of K-Cl cotransport in a nonmammalian, nonerythroid cell.

METHODS

Preparation of *Xenopus laevis* oocytes. Adult female normal and albino *X. laevis* frogs were purchased from Carolina Biological Supply (Burlington, NC) and maintained at the Institution animal facility under constant control of room temperature and humidity at 16°C and 65%, respectively. Frogs were fed with frog brittle dry food from Carolina Biological Supply, and water was changed twice a week. Oocytes were surgically collected from anesthetized animals under 0.17% tricaine and incubated for 1 h with vigorous shaking in ND96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES/Tris, pH 7.4) and 2 mg/ml of collagenase B, after which oocytes were washed four times in ND96 and manually defolliculated. Stage V–VI oocytes (11) were incubated for 2–4 days in ND96 at 18°C supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. The incubation medium was changed every 24 h. The day of the influx experiment, oocytes were switched to a Cl⁻-free ND96

(in mM: 96 Na⁺ isethionate, 2 K⁺-gluconate, 1.8 Ca²⁺-pyruvate, 1 Mg²⁺-gluconate, 5 HEPES, 2.5 sodium pyruvate, and 5 mg% gentamicin, pH 7.4) for 2 h before the assay.

Assessment of K-Cl cotransporter function. Functional analysis of the K-Cl cotransporter consisted of measuring tracer ⁸⁶Rb⁺ uptake (New England Nuclear) in groups of at least 15 oocytes. ⁸⁶Rb⁺ uptake was measured under both isotonic and hypotonic conditions with the following general protocol: a 30-min incubation period in a hypotonic K⁺ and Cl⁻-free medium (in mM: 50 *N*-methyl-D-glucamine (NMDG)-gluconate, 4.6 Ca²⁺-gluconate, 1.0 M glucose, 5 HEPES/Tris, pH 7.4) with 1 mM ouabain, followed by a 60-min uptake period in a hypotonic Na⁺-free and KCl-containing medium with variable K⁺ and Cl⁻ concentrations (in mM: 0–50 NMDG-Cl, 0–50 KCl, 0–50 NMDG-gluconate, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4), supplemented with 1 mM ouabain, and 2.5 μCi of ⁸⁶Rb⁺. Experiments in isotonic conditions were performed using the same solutions but supplemented with sucrose at 3.5 μ/100 ml to increase the osmolality of the solutions to the isosmolar conditions for oocytes (~210 mosmol/kgH₂O). Ouabain was added to prevent ⁸⁶Rb⁺ uptake via the Na⁺-K⁺-ATPase. The absence of extracellular Na⁺ and the hypotonicity of the uptake medium prevented ⁸⁶Rb⁺ uptake or ⁸⁶Rb⁺-K⁺ exchange by the endogenous Na-K-2Cl cotransporter that is present in oocytes (13).

All uptakes were performed at room temperature. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isotopy to remove extracellular tracer. Oocytes were dissolved in 10% sodium dodecyl sulfate, and tracer activity was determined for each oocyte by beta scintillation counting.

To determine the ion transport kinetics of the K-Cl cotransporter we performed experiments using varying concentrations of K⁺ and Cl⁻. To maintain osmolality and ionic strength, gluconate was used as a Cl⁻ substitute and NMDG as a K⁺ substitute. The sensitivity and kinetics for several inhibitors were assessed by exposing groups of oocytes to inhibitor at concentrations varying from 20 μM to 2 mM. For these experiments, the desired concentration of the inhibitor was present during both the incubation and uptake periods. We also assessed the effect of several drugs on the activation of the K-Cl cotransporter by adding the drug during 15 min before the uptake period.

RT-PCR amplification of KCCs. A BLAST search of the National Center for Biotechnology expressed sequence tag (EST) database revealed a 549-bp coding sequence EST (GenBank accession no. AW646505) from *X. laevis* oocytes with significant homology to the mammalian KCCs, and we amplified this 549-bp fragment from *Xenopus* oocyte total RNA by RT-PCR. *Xenopus* KCC oligonucleotide primers were designed using the EST sequence. The sense primer 5'-ACAGTACTCTCGGAGACTACC-3' and antisense primer 5'-GATACGTAATGATTAAGAAAGG-3' amplified a fragment of 528 bp. The amplified band spans a region of the KCC proteins that is encoded by three exons in a *Drosophila* homologue (see GenBank accession no. AE003462) and all four mammalian KCCs (D. B. Mount, unpublished data). Given the level of genomic conservation between this region of the KCC genes in mammals and *Drosophila*, a similar genomic structure is likely for this KCC gene in *X. laevis*, such that the PCR primers used for the initial RT-PCR reaction will amplify a larger DNA fragment from contaminating genomic DNA, if at all. However, reverse transcription was omitted in control samples to verify that contaminating genomic DNA was not amplified in the RT-PCR samples. To increase the specificity of the PCR amplification, nested PCR of the amplified band was performed with two

internal primers that amplify a band of 359 bp; the sense primer for nested PCR was 5'-AGCAGGACAGGCACTGAAA-CAC-3' and the antisense primer was 5'-GGAAGGGCA-GAAGCATAAAGC-3'. A second overlapping EST (GenBank accession no. BE576764) was subsequently identified, obtained from Research Genetics, and sequenced in entirety using fluorescent dye termination chemistry (BigDye, Applied Biosystems).

Total RNA was extracted from defolliculated oocytes and other tissues using the guanidinium isothiocyanate-cesium chloride method (49). PCR was performed with 1 μ l of reverse-transcribed RNA in 20- μ l reactions containing 1 \times PCR buffer (in mM: 10 Tris-HCl, 1.5 MgCl₂, 50 KCl, pH 8.3), 0.1 mM of each dNTP, 10 μ M of each primer, and one unit of *Taq* DNA polymerase (Life Technologies). Thirty-five PCR cycles were performed with the following profile: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The last cycle was followed by a final extension step of 5 min at 72°C.

The PCR product from oocytes was gel purified from a 1.5% agarose gel and sequenced by the dideoxy chain termination method (50) using the Sequenase DNA sequencing kit (USB). Once the nature of the single PCR band from oocytes was confirmed by DNA sequencing as a *Xenopus* homologue of the mammalian KCC cotransporters (see results), Southern blot of all tissue PCR products was performed under high-stringency conditions using the 528-bp fragment to generate a nonradioactive probe by using the PCR DIG probe synthesis kit (Boehringer Mannheim, Germany). Hybridization bands were detected by an immunoperoxidase reaction.

Statistical analysis. Statistical significance is defined as two-tailed $P < 0.05$, and the results are presented as means \pm SE. The significance of the differences between groups was tested by one-way ANOVA with multiple comparison using Bonferroni correction or by the Kruskal-Wallis one-way analysis of variance on ranks with the Dunn's method for multiple comparison procedures, as needed.

RESULTS

Expression of an endogenous K-Cl cotransporter in *Xenopus* oocytes. Figure 1 shows a summary from

seven experiments in which $^{86}\text{Rb}^+$ uptake was assessed using an uptake solution containing 2 mM and 50 mM of extracellular K^+ and Cl^- , respectively. Uptakes were performed under both isotonic conditions (220 mosmol/kgH₂O for *Xenopus* oocytes) and hypotonic conditions (110 mosmol/kgH₂O). Under isotonic conditions, oocytes exhibited a Rb^+ uptake of 10.1 ± 1.4 pmol-oocyte⁻¹·h⁻¹ that was reduced to 6.3 ± 1.3 pmol-oocyte⁻¹·h⁻¹ when oocytes were incubated in Cl^- -free medium. However, the difference did not reach significance. Under hypotonic conditions, Rb^+ uptake in the presence of extracellular Cl^- increased to 52.5 ± 11.0 pmol-oocyte⁻¹·h⁻¹ ($P < 0.00001$); the increased Rb^+ uptake was Cl^- dependent, since uptake under hypotonic Cl^- -free conditions was 5.22 ± 1.0 pmol-oocyte⁻¹·h⁻¹ ($P < 0.001$). Therefore, oocytes exhibit a Cl^- -dependent $^{86}\text{Rb}^+$ uptake mechanism that is activated by cell swelling (hypotonic conditions). A similar observation was made using oocytes harvested from the albino type of *X. laevis* (data not shown). In addition, Fig. 1, inset, shows the result of a single experiment in which $^{36}\text{Cl}^-$ uptake was assessed in the presence of 10 mM and 50 mM of extracellular K^+ and Cl^- , respectively. In isotonicity, $^{36}\text{Cl}^-$ uptake was similar in the presence or absence of extracellular K^+ [908 ± 124 vs. 912 ± 346 pmol-oocyte⁻¹·h⁻¹, P = nonsignificant (NS)]. In contrast, incubation in hypotonicity induced an increase in $^{36}\text{Cl}^-$ uptake to $2,960 \pm 455$ pmol-oocyte⁻¹·h⁻¹ ($P < 0.01$) that was partially but significantly K^+ -dependent ($1,615 \pm 296$ pmol-oocyte⁻¹·h⁻¹, $P < 0.01$). Thus oocytes also exhibit a K^+ -dependent $^{36}\text{Cl}^-$ uptake mechanism that is only apparent during cell swelling.

K-Cl cotransport in several cells is uniquely activated by the *N*-alkylating agent *N*-ethylmaleimide (NEM) (32), whereas Na-K-2Cl cotransport is inhibited by this agent (55). We therefore analyzed the effect of

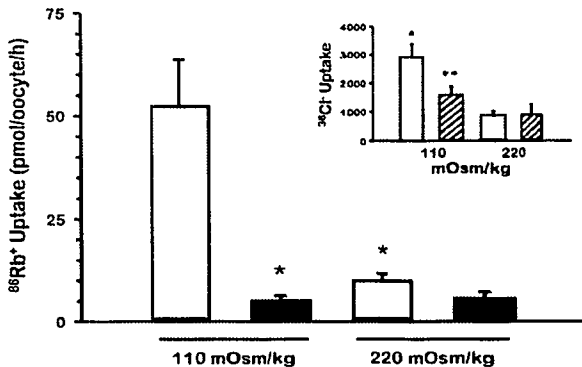


Fig. 1. K-Cl cotransport activity in *Xenopus laevis* oocytes. $^{86}\text{Rb}^+$ uptakes were measured under hypotonic or isotonic conditions, as indicated, in the presence of 2 mM K^+ with (open bars) or without (filled bars) extracellular Cl^- . Each bar represents mean \pm SE of 7 experiments from different frogs. *Significantly different from uptake in control group at 110 mosmol/kgH₂O ($P < 0.001$). Inset: a single experiment in which $^{36}\text{Cl}^-$ uptake was assessed in hypotonic or isotonic conditions, in the presence (open bars) or absence (hatched bars) of 10 mM extracellular K^+ . For $^{36}\text{Cl}^-$ uptake experiment, oocytes were exposed to a 30-min incubation period in a hypotonic or isotonic K^+ and Cl^- -free medium, followed by 30-min uptake period in an isotonic or hypotonic Na^+ -free medium in the presence of 1 $\mu\text{Ci/ml}$ of H^{36}Cl . * $P < 0.01$ vs. uptake in 220 mosmol/kgH₂O. ** $P < 0.01$ vs. uptake in control group in 110 mosmol/kgH₂O.

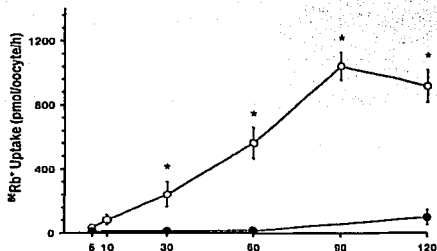
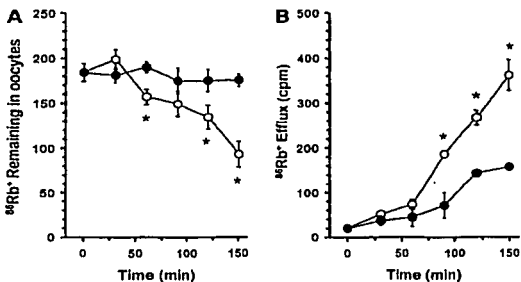


Fig. 2. Time course (in min) of $^{86}\text{Rb}^+$ influx in *X. laevis* oocytes. Oocytes were incubated for 30 min in K^+ and Cl^- -free hypotonic medium and then in a 10 mM K^+ -containing medium in the presence (○) or absence (●) of 50 mM Cl^- . Uptake periods are indicated. Each point represents mean \pm SE of 15 oocytes. * $P < 0.001$ vs. Cl^- -free group.

NEM upon $^{86}\text{Rb}^+$ uptake in oocytes incubated in isotonic conditions. In the presence of 10 mM K^+ and 50 mM Cl^- , exposing oocytes to 1 mM NEM before the influx period increased the $^{86}\text{Rb}^+$ uptake by ~ 2 -fold from 117 ± 6.3 pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$ in the control group to 227 ± 29 pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$ in the NEM-treated group ($P < 0.001$). The increased uptake induced by NEM was Cl^- dependent. In the absence of extracellular Cl^- , influx was similar between the control and NEM-treated oocytes (68 ± 7 vs. 60 ± 11 pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$, $P = \text{NS}$). Therefore, under isotonic conditions, addition of NEM resulted in increased activity of the K-Cl cotransporter.

Figure 2 illustrates the time course of $^{86}\text{Rb}^+$ uptake when oocytes were exposed to a hypotonic uptake medium containing 10 mM of extracellular K^+ , in the presence or absence of 50 mM Cl^- . When Cl^- was present in the extracellular medium we observed increased $^{86}\text{Rb}^+$ uptake that was linear for 90 min. This uptake was due to the K-Cl cotransporter since no uptake was observed in the Cl^- -free uptake medium.



The K-Cl cotransporter is one of the efflux pathways that are activated by cell swelling, as part of the regulatory volume decrease (RVD) response. With this in mind, we also examined $^{86}\text{Rb}^+$ efflux when oocytes were exposed to hypotonicity (Fig. 3). Over a 150-min period we observed a progressive reduction in the amount of $^{86}\text{Rb}^+$ remaining in the cells, together with a gradual increase in the tracer Rb^+ in the extracellular medium. As shown in Fig. 3, A and B, the addition of the loop diuretic furosemide significantly reduced the efflux of $^{86}\text{Rb}^+$ from the cells, indicating the proportion of efflux that was through the K-Cl cotransporter.

Kinetics properties and anion dependence of the oocyte K-Cl cotransporter. Figure 4 shows the Rb^+ and Cl^- dependency of the K-Cl cotransporter in *Xenopus* oocytes. To assess the Rb^+ kinetics, uptakes were performed in extracellular media with fixed concentration of Cl^- at 50 mM, changing concentrations of Rb^+ from 0 to 50 mM. In contrast, to assess the Cl^- kinetics, uptakes were done with a fixed concentration of K^+ at 20 mM, changing the concentration of Cl^- from 0 to 50. As illustrated in Fig. 4, Rb^+ uptake showed Michaelis-Menten behavior. The calculated Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) for extracellular Rb^+ concentration were 27.7 ± 3.0 mM and $1,531 \pm 78$ pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$, respectively, and the apparent K_m and V_{max} values for extracellular Cl^- concentration were 15.4 ± 4.7 mM and 318 ± 39 pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$, respectively. Consistent with electroneutrality of the transport process, the Hill coefficient for both ions remained close to unity: 1.04 ± 0.17 and 1.07 ± 0.14 for K^+ and Cl^- , respectively.

It has been shown that some extracellular anions other than Cl^- can support ion translocation through the K-Cl cotransporter of sheep and human erythrocytes (43). It was thus of interest to study the anion series for the oocyte K-Cl cotransporter, assessed as the relative amount of $^{86}\text{Rb}^+$ influx in the presence of anions other than Cl^- . We observed no significant difference in tracer $^{86}\text{Rb}^+$ uptake in the presence of Cl^- (129 ± 18 pmol \cdot oocyte $^{-1}\cdot$ h $^{-1}$), PO_4^{3-} (130 ± 21

Fig. 3. $^{86}\text{Rb}^+$ efflux in *X. laevis* oocytes. A: single experiment showing time course of tracer Rb^+ remaining in oocytes (pmol/oocytes). B: mean of 3 experiments showing time course for appearance of $^{86}\text{Rb}^+$ in the extracellular medium (counts per min (cpm)). For these experiments, oocytes were incubated for 1 h in regular isotonic ND96 containing $5.0 \mu\text{Ci/ml}$ of $^{86}\text{Rb}^+$. At the end of this loading period, oocytes were washed 5 times and transferred to a hypotonic solution containing (in mM) 48 *N*-methyl-D-glucamine gluconate, 2 KCl, 1.5 CaCl $_2$, 1 MgCl $_2$, 5.0 HEPES, and 1 ouabain, without extracellular $^{86}\text{Rb}^+$, in the absence (○) or presence (●) of 2 mM extracellular furosemide. * $P < 0.01$ vs. same time point in presence of furosemide. In A each point represents mean \pm SE of 15 oocytes. In B each point represents mean \pm SE of 3 experiments.

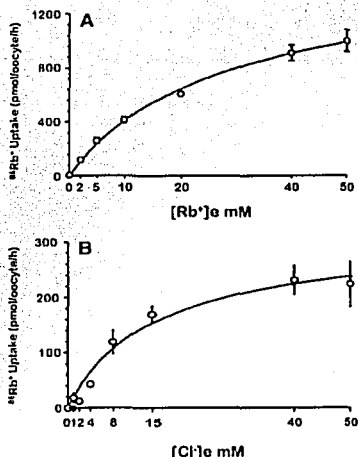


Fig. 4. Kinetic analysis of Rb⁺ uptake in *Xenopus* oocytes. A: Rb⁺ dependency of Rb⁺ uptake. Uptakes were performed over a 60-min period in 110 mosmol/kgH₂O with a fixed concentration of Cl⁻ at 50 mM, changing extracellular concentration of Rb⁺ ([Rb⁺]_o) from 0 to 50 mM. B: Cl⁻ dependency of Rb⁺ uptake. Uptakes were performed over a 60-min period in 110 mosmol/kgH₂O with a fixed concentration of K⁺ at 20 mM and changing extracellular concentration of Cl⁻ ([Cl⁻]_o) from 0 to 50 mM. For data on A, uptakes were also measured in groups of oocytes in the absence of Cl⁻ (data not shown), and the mean values for these groups were subtracted in corresponding RbCl-containing groups to analyze only the Cl⁻-dependent Rb⁺ uptake. For data on B, the uptake observed in a solution containing 20 mM K⁺ and 0 mM Cl⁻ was subtracted from uptakes in all other groups. Lines were fit using the Michaelis-Menten equation. The Hill coefficients for K⁺ and Cl⁻ were close to unity: 1.04 ± 0.16 and 1.07 ± 0.14 for K⁺ and Cl⁻, respectively. Each point represents the mean ± SE of 15 oocytes.

pmol/oocyte⁻¹·h⁻¹), or Br⁻ (101 ± 16 pmol/oocyte⁻¹·h⁻¹), whereas a significant reduction ($P < 0.001$) was observed in the presence of I⁻ (67 ± 15 pmol/oocyte⁻¹·h⁻¹), SCN⁻ (44 ± 12 pmol/oocyte⁻¹·h⁻¹), and gluconate (25 ± 5 pmol/oocyte⁻¹·h⁻¹). The function profile in the presence of different anions of the oocyte transporter was thus Cl⁻ = PO₄³⁻ = Br⁻ > I⁻ > SCN⁻ > gluconate.

Sensitivity to inhibitors. Figure 5 illustrates the effect of a 100 μM concentration of several inhibitors of cation-chloride cotransporters on the oocyte K-Cl cotransporter. At a 100 μM concentration, DIDS had no effect on ⁸⁶Rb⁺ uptake, whereas the mammalian KCCs are sensitive to DIDS at this concentration (32, 38, 42). The addition of a 100 μM concentration of the loop diuretic furosemide or bumetanide to the uptake medium resulted in a 22 and 20% reduction in the Cl⁻-dependent tracer Rb⁺ uptake, respectively, compared

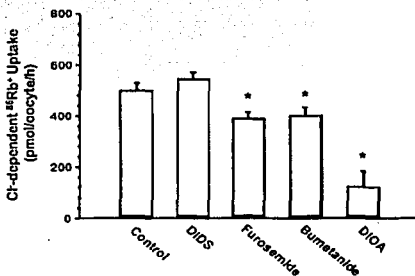


Fig. 5. Effect of transport inhibitors on Cl⁻-dependent ⁸⁶Rb⁺ uptake. Uptakes were assessed in 10 mM K⁺ and 50 mM Cl⁻-hypotonic medium. In the Cl⁻-free group gluconate was used as substitute. The concentration of all inhibitors was 100 μM. * $P < 0.05$ vs. uptake in control conditions (1st bar). Each bar represents mean ± SE of 15 oocytes.

with uptake observed in control group. The reduction was statistically significant ($P < 0.001$). In contrast, a 100 μM concentration of [(dihydroindenyl)oxy]alkanoic acid (DIOA) reduced the uptake by 76% ($P < 0.000001$). Thus the oocyte K-Cl cotransporter is more sensitive to DIOA than to loop diuretics, as is the case for the mammalian isoforms (38). Figure 6 shows the concentration-dependent inhibition of the oocyte K-Cl cotransporter by loop diuretics. The IC₅₀ values for furosemide and bumetanide were calculated at 200 and 500 μM, respectively. Although the four mammalian KCCs differ in relative sensitivity to the two loop

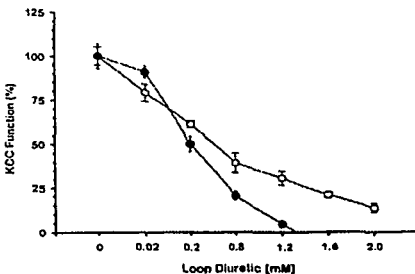


Fig. 6. Kinetic analyses of K-Cl cotransporter (KCC) inhibition by loop diuretics. Uptakes were assessed in hypotonic medium. Groups of 10 *Xenopus* oocytes were exposed to increasing concentrations of furosemide (●) or bumetanide (○) in the preincubation and uptake medium, from 20 to 2,000 μM. Data were normalized as the percentage of uptake, taking 100% as the value in the absence of loop diuretics. The calculated IC₅₀ was 200 μM and 500 μM for furosemide and bumetanide, respectively. Each point represents the mean ± SE of 15 oocytes.

diuretics, bumetanide is always less effective than furosemide, as is the case for the oocyte K-Cl cotransporter.

Regulation of the oocyte K-Cl cotransporter. In red blood cells of several species, swelling-induced activation of K-Cl cotransport appears to involve a protein dephosphorylation step, presumably of the transporter protein itself (10). With this in mind, we assessed the functional effect of inhibiting the protein phosphatases. To discriminate between phosphatases, we used 100 nM of calyculin A, which inhibits the function of both protein phosphatases 1 and 2A (PP1 and PP2A), 1 nM of okadaic acid, a concentration that inhibits only PP2A (4), and 100 pM cypermethrin, which inhibits the function of protein phosphatase 2B (PP2B) (12). As Fig. 7 shows, the increased $^{86}\text{Rb}^+$ uptake induced by hypotonicity was completely abrogated by calyculin A. In contrast, no effect was observed with okadaic acid and cypermethrin.

Many ion transporters are dramatically affected by exposure to Hg^{2+} (22, 36, 57). For example, orthologues of the basolateral isoform of the Na-K-2Cl cotransporter exhibit variable inhibition by mercury (22), whereas other proteins such as aquaporin-6 are activated by Hg^{2+} (60). We thus analyzed the effect of Hg^{2+} on the $^{86}\text{Rb}^+$ uptake of *Xenopus* oocytes. As Fig. 8 shows, oocyte incubation in the presence of 160 μM HgCl_2 under isotonic conditions for the 15 min previous to the influx period resulted in a dramatic increase in $^{86}\text{Rb}^+$ uptake. We used this HgCl_2 concentration since this was the dose at which maximal response was observed. This increase was completely prevented by pretreatment of oocytes with 10 mM of the reducing agent dithiothreitol (DTT) (22). The incubation of oocytes with and without extracellular Cl^- during uptake

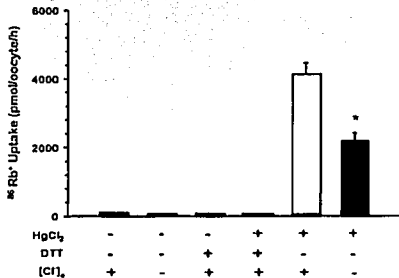


Fig. 8. Effect of 160 μM HgCl_2 and 10 mM dithiothreitol (DTT) on $^{86}\text{Rb}^+$ uptake in *Xenopus* oocytes. Cells were incubated in isotonic conditions during the uptake. Before the uptake period, oocytes were exposed to 30 min of DTT alone, 15 min of HgCl_2 alone, or 30 min of DTT plus HgCl_2 in the last 15 min; HgCl_2 or DTT was not present during the uptake period. As indicated, uptakes were performed in the presence or absence of extracellular Cl^- . Each point represents the mean \pm SE of 30 oocytes. * $P < 0.001$ vs. HgCl_2 control group.

revealed that HgCl_2 -induced increase in $^{86}\text{Rb}^+$ uptake is composed of at least two distinct pathways, each accounting for $\sim 50\%$ of the total uptake. One pathway is Cl^- dependent, consistent with K-Cl cotransport, whereas the other is Cl^- independent, suggesting the opening of a cation channel.

Xenopus oocytes express a homologue of the mammalian KCCs. A BLAST search of EST databases revealed the existence of one *X. laevis* EST (accession no. AW646505) from oocytes that was homologous to KCC sequences, exhibiting 76% identity to the rat KCC1 sequence. On the basis of this *Xenopus* EST, we designed a primer pair to amplify a fragment of 528 bp (see METHODS). A single band of the expected size was amplified whereas no band was observed in the absence of reverse transcription (data not shown). A second primer pair was used for nested PCR-amplifying of 349 bp using the first band as a template. In addition, the 528-bp PCR band was gel purified and the DNA sequence was confirmed. A second overlapping EST was subsequently identified, obtained from Research Genetics, and sequenced in entirety; the composite cDNA (accession no. AF326505) encodes the COOH-terminal 358 amino acids of the *Xenopus* KCC and the entire 3'-untranslated region. Figure 9 shows the alignment of the deduced amino acid sequence of each of the mammalian KCC cDNAs with the partial sequence of the *Xenopus* KCC; this COOH-terminal fragment exhibits 68% identity with rat KCC1 (accession no. U55816), 56% with rat KCC2 (accession no. U55816), 76% with human KCC3 (accession no. AF105366), and 62% with mouse KCC4 (accession no. AF087436). We also performed RT-PCR and Southern blot analysis of RT-PCR products obtained from several tissues, using the 528-bp product from oocytes as a nonradioactive probe. Figure 10A shows the amplifica-

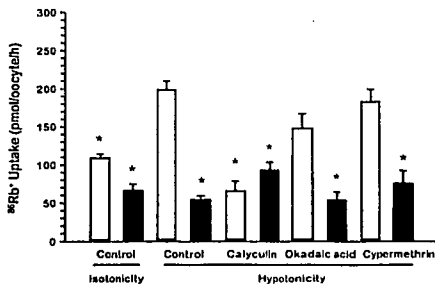


Fig. 7. Effect of protein phosphatase inhibition upon the hypotonicity-induced activation of the K-Cl cotransporter in *Xenopus* oocytes. Influxes were performed under isotonic or hypotonic conditions, exposing oocytes to calyculin A, okadaic acid, or cypermethrin during preincubation and uptake periods as stated. Uptakes were assessed in 10 mM K^+ -containing medium in the presence (open bars) and absence (filled bars) of extracellular Cl^- . * $P < 0.001$ vs. uptake in control conditions in hypotonicity.

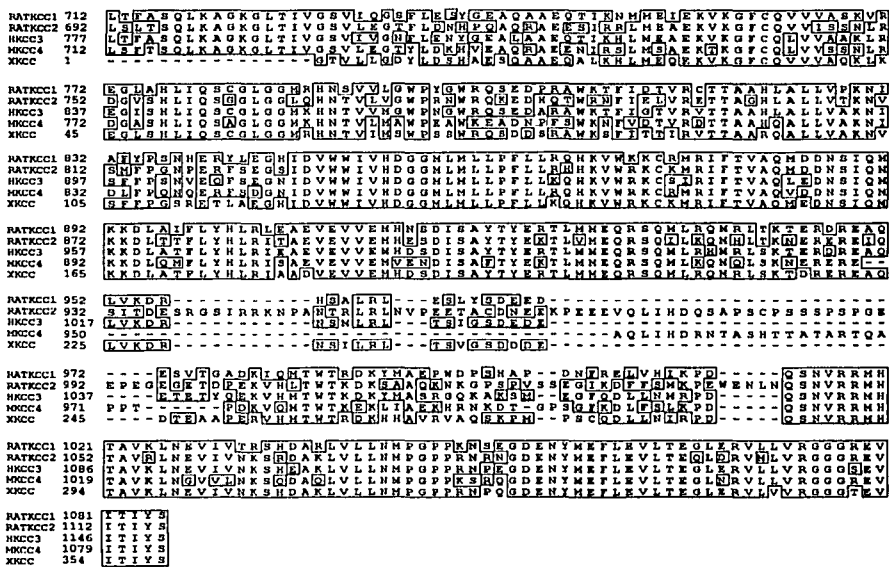


Fig. 9. Protein alignments of the mammalian K-Cl cotransporters KCC1 to KCC4, with the deduced amino acid sequence of the *Xenopus* oocyte K-Cl cotransporter (XKCC). The fragment corresponds to a part of the carboxy terminus, which is predicted to be cytoplasmic. Identical segments are boxed. HKCC3, human KCC3; MKCC4, mouse KCC4.

tion of a ~528-bp product from all tested tissues. Control PCR reactions in the absence of reverse transcripts were negative for all tissues (data not shown, except for oocytes mRNA in last lane of Fig. 10A), indicating that the amplified product was not due to contamination with genomic DNA. In addition, Fig. 10B shows that the PCR product obtained from all tissues was able to hybridize with the KCC-specific probe on a Southern blot of an agarose gel.

DISCUSSION

In the present study we have defined the principal functional, pharmacological, and regulatory properties of the K-Cl cotransport pathway that is expressed in *X. laevis* oocytes. Under isotonic conditions, some individual experiments (e.g., Fig. 7) showed a small but significant Cl⁻-dependent ⁸⁶Rb⁺ uptake. This is potentially due to weak volume-independent activation of the K-Cl cotransporter; however, experimental variability at this low level of transport may also play a role. In addition, this is theoretically due to activity of

the oocyte Na-K-2Cl cotransporter, since Lytle et al. (35) have proposed that in the absence of external Na, as was the case in our experiments, the Na-K-2Cl cotransporter can potentially catalyze Cl⁻-dependent K/Rb exchange. Regardless, when data from multiple experiments are analyzed (Fig. 1), the difference between isotonic Rb⁺ uptake in the presence and absence of Cl⁻ does not reach statistical significance. Under hypotonic conditions, oocytes exhibited a very significant Cl⁻-dependent ⁸⁶Rb⁺ uptake. In these circumstances, it is particularly unlikely that ⁸⁶Rb⁺ transport activity in the absence of Na⁺ was due to the Na-K-2Cl cotransporter, because this cotransporter in oocytes is inhibited by hypotonicity (13). In addition, oocytes exhibit a K⁺-dependent ³⁶Cl⁻ uptake pathway that is activated by hypotonicity. The Cl⁻-dependent ⁸⁶Rb⁺ uptake in oocytes exhibited the following characteristics: 1) transport can be activated by cell swelling and to a lesser extent by NEM, which are the classic activators of K-Cl cotransport in several cell types and species (32); 2) the transport of K⁺ and Cl⁻ exhibits

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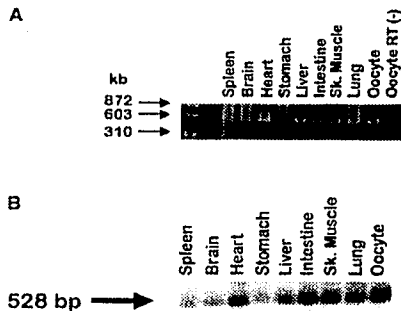


Fig. 10. *A*: An acrylamide gel of RT-PCR products from several *Xenopus* tissues, using a KCC-specific primer pair to amplify a 528-bp fragment. The last lane shows the control PCR from oocyte RNA in the absence of reverse transcriptase (RT-). *B*: Southern blot analysis of RT-PCR products obtained from total RNA extracted from several *X. laevis* tissues. Membranes were probed under high-stringency conditions with a probe constructed from the 528-bp fragment of the *Xenopus* K-Cl cotransporter.

interdependency and electroneutrality, with Hill coefficients of 1; 3) the K-Cl cotransporter is sensitive to loop diuretics, with higher affinity for furosemide over bumetanide, a common feature of the K-Cl cotransporters (16, 44); 4) it is also sensitive to the specific K-Cl inhibitor DIOA (15); and 5) activation by hypotonicity can be prevented by the protein phosphatase inhibitor calyculin A. At the molecular level, we have identified a partial cDNA clone that encodes a protein with a high degree of identity (>75%) with mammalian KCC sequences. Although we show no direct evidence that this gene is responsible for the Cl^- -dependent $^{86}\text{Rb}^+$ uptake observed in this study, our data clearly indicate that *Xenopus* oocytes express a K-Cl cotransporter that shares functional and molecular properties with the mammalian KCCs.

To be fertilized, the *Xenopus* female lays the oocytes into pond water of very low osmolality. Because of still not very well understood mechanisms that include conformational changes of the cytoskeleton, when oocytes become fertilized eggs they develop a complete resistance to cell swelling. Kelly et al. (28) showed that frog fertilized eggs transferred to dilute buffer with osmolality of 10 mosmol/kg H_2O for several hours developed no changes in cell volume, whereas oocytes exhibit a slow increase in cell volume over time and eventually burst. Since it was shown in the same study that oocytes do not develop a clear RVD response, the authors suggested that in oocytes exposed to dilute buffer osmolyte efflux occurs and limits swelling. Thus oocytes clearly possess the transport mechanisms to release intracellular ions to reduce cell swelling while

they become fertilized eggs, since they express a swelling-activated K-Cl cotransporter that is capable of K-Cl efflux (Fig. 3).

Kinetic analysis of the $^{86}\text{Rb}^+$ uptake in swollen oocytes showed that both K^+ and Cl^- are required. The Hill coefficients for both ions were close to unity, indicating an electroneutral transport process with a stoichiometry of 1:1. The affinity for extracellular ions revealed an apparent K_m for extracellular K^+ of ~ 22 mM and for extracellular Cl^- of ~ 15 mM. These values are similar to those of the mammalian KCC1 isoform (38). Whereas all of the KCC isoforms studied thus far exhibit similar affinity for extracellular Cl^- (14 to 17 mM), they differ dramatically in the affinity for extracellular K^+ . The isoform with the highest K^+ affinity is rat KCC2, with a K_m for extracellular K^+ of ~ 5 mM (42), followed by KCC4 (K_m of ~ 17 mM) (38), KCC1 (K_m of ~ 25 mM) (38), and KCC3 (K_m of ~ 51 mM) (37). Although the direction of transport is ultimately dictated by gradients for the transported ions, it has been proposed that KCCs with a higher cation affinity (KCC2 and KCC4) can transport K^+ in both directions at higher rates. This has been verified experimentally in neurons, which predominantly express KCC2 (23). In contrast, isoforms with lower K^+ affinity (KCC1 and KCC3) are more suited to function as extrusion mechanisms, when the gradient for K^+ will favor efflux (42).

Despite the lack of variation in Cl^- affinity, K-Cl cotransporters differ in the anion series of rubidium transport, i.e., the relative activity in the presence of anions other than Cl^- (37). The *Xenopus* transporter is similar in this respect, in that anions other than Cl^- could also support K^+ translocation. In fact, the anion series of the endogenous oocyte K-Cl cotransporter is very similar to the anion series observed in KCC1-injected oocytes (38).

As shown for K-Cl cotransport in other species, the *Xenopus* transporter is sensitive to loop diuretics and other inhibitors of anion transport (16, 20, 42). In addition, $^{86}\text{Rb}^+$ uptake was also sensitive to DIOA, which appears to be a specific inhibitor of K-Cl cotransport (15); this compound has no effect on the oocyte Na-K-2Cl cotransporter (data not shown).

Inhibition of protein phosphatases prevents the swelling- and NEM-induced activation of the K-Cl cotransporter in several cells (6, 25, 27, 29, 52), and it is widely accepted that dephosphorylation is required for activation of the cotransporter. Consistent with the data from other cells, we observed in the present study that the protein phosphatase inhibitor calyculin A abolishes hypotonic activation of the oocyte cotransporter (Fig. 8). Calyculin A inhibits two types of phosphatase, both PP1 and PP2A (9). To determine the phosphatase involved, we tested the effect of okadaic acid, which is a specific PP2A inhibitor at the 1 nM concentration used, and the specific PP2B inhibitor cypermethrin (4, 12); neither inhibitor affected the activation of the cotransporter by hypotonicity. Therefore, it is likely that in *Xenopus* oocytes, PP1 is the

phosphatase involved in activation of the K-Cl cotransporter during cell swelling (6, 25).

Several cotransporters are known to be affected by exposure to mercury (Hg^{2+}). For example, transporter for the NaSi cotransporter (36) and the NaPi-3 cotransporters in *Xenopus* oocytes is significantly reduced when oocytes are exposed to Hg^{2+} (57). Similarly, heterologous expression of the basolateral isoform of the Na-K-2Cl cotransporter in HEK-293 cells is sensitive to Hg^{2+} (22). In contrast, when expressed in *Xenopus* oocytes, aquaporin-6 is activated by this heavy metal (60). It is known that Hg^{2+} affects the function of channels and transporters by interacting with sulfhydryl groups (-SH) on cysteine residues (7, 60), and in some cases this has been proven by mutagenesis studies (30, 41). In the present study we observed that exposing *Xenopus* oocytes to 150 μ M HgCl₂ resulted in increased $^{86}Rb^{+}$ uptake by activation of at least two pathways: one that is Cl^{-} dependent and another that is Cl^{-} independent. Because uptakes were performed in the absence of extracellular Na^{+} , it is unlikely that the opening of this Cl^{-} dependent $^{86}Rb^{+}$ influx pathway represents activity of the Na-K-2Cl cotransporter. In addition, Jacoby et al. (22) have shown that Na-K-2Cl cotransporter is inhibited by Hg^{2+} . Therefore, the Cl^{-} dependent fraction of the Hg^{2+} -induced increase in $^{86}Rb^{+}$ uptake is due to activation of K-Cl cotransport. The mechanism by which Hg^{2+} affects the function of membrane proteins is not clear, but it is known that Hg^{2+} interacts with cysteinyl sulfhydryls (-SH). The observation that the effect of Hg^{2+} on $^{86}Rb^{+}$ influx in oocytes was completely prevented by the reducing agent DTT suggests that, indeed, interaction of Hg^{2+} with SH groups is necessary for the stimulatory effect. Of interest, although it has been suggested that NEM also affects the K-Cl cotransporter function by interaction with SH groups, the stimulatory effect of NEM upon the K-Cl cotransporter observed in this study (Fig. 2) was significantly smaller than the effect of Hg^{2+} . It is still unclear, however, if the activating effect of NEM on the cotransporter is related to NEM-induced dephosphorylation or direct modification of SH groups. There are reports supporting both possibilities (24, 31).

Finally, we have confirmed the expression of a KCC isoform in *Xenopus* oocytes and multiple other tissues. Sequence data from a *Xenopus* EST was used to clone a partial cDNA by RT-PCR, which overlapped with another fully sequenced EST cDNA. The predicted protein sequence of this partial cDNA was more homologous to the KCC1-KCC3 subfamily than to the KCC2-KCC4 subfamily of the K-Cl cotransporters. Cloning of the full-length cDNA will be pursued, since this will provide the means for structure-function studies of this KCC and for characterization of the physiological role(s) of this transporter in *Xenopus* tissues.

In summary, we have shown that *X. laevis* oocytes express a K-Cl cotransporter in the plasma membrane that is activated by cell swelling, NEM, and $HgCl_2$ and inhibited by loop diuretics and DIOA. The functional

properties resemble those of mammalian KCC1, and the sequence of the COOH terminus is closest to KCC3, indicating that this *Xenopus* KCC is the amphibian orthologue of one or both of these low-affinity K-Cl cotransporters.

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