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MECANISMO DE ACCION DE LOS RECEPTORES ALFA$_1$ - ADRENERGICOS

T E S I S

Que para obtener el Grado de

Maestra en Investigación Biomedica

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I N D I C E

I. INTRODUCCION

1. GENERALIDADES

1.1 Importancia de las catecolaminas en el sistema neuroendócrino.

El sistema neuroendócrino es el encargado de la integración del funcionamiento normal tanto de los vertebrados como de los invertebrados. El mecanismo por el cual se regulan y coordinan la mayor parte, sino es que todas sus funciones, es mediante la comunicación intercelular, ya sea a través de las neuronas neurosecretoras, por el sistema nervioso o bien por una glándula endócrina como tal. Esta comunicación se efectúa por una serie de mensajeros químicos (neurohormonas, neurotrasmisores u hormonas) que son las encargadas del control de un gran número de eventos en las células blanco.

Las aminas adrenérgicas son un grupo de substancias cuyos representantes endógenos, la epinefrina y la norepinefrina, actúan como hormonas y/o neurotransmisores. La importancia de estos agentes radica en la gran variedad de procesos que son capaces de regular. La epinefrina parece ser la hormona de las "grandes urgencias" puesto que es liberada en casos de estrés, preparando al animal para la lucha o la huída. Estos agentes tienen efectos desde el músculo cardiaco, hasta la regulación de un gran número de vías metabólicas como la glucogenólisis, la ureogénesis y la lipólisis, pero además pueden mediar la liberación de otras hormonas o neurotransmisores. De aquí la importancia del estudio de la síntesis, secreción, efectos fisiológicos y mecanismo de acción de estos agentes.

1.2 Receptores Adrenérgicos.

Las catecolaminas como prácticamente todas las hormonas que actúan a nivel de la membrana plasmática promueven la señal hormonal interactuando con sus receptores específicos. Estas moléculas son proteínas, en la mayoría de los casos localizadas en la membrana plasmática, que tienen la capacidad de unir específicamente ligandos endógenos como hormonas, neurotransmisores y autacoides. El complejo hormona-receptor está acoplado a un sistema transductor localizado también en la membrana plasmática que promueve la generación de una señal a través de la síntesis o liberación de otra sustancia efectora o segundo mensajero el cual actúa alterando la velocidad de rutas metabólicas ya establecidas.

En 1948 Ahlquist (1) clasificó los efectos de las catecolaminas en dos grupos de acuerdo con su orden de potencia concluyendo que estos agentes podían interactuar con dos tipos de receptores a los que denominó "alfa" y "beta", Robinson et al en 1967 asociaron los efectos beta adrenérgicos a la activación de la adenilato ciclasa (2) y este mismo grupo (3) propuso que los efectos alfa eran debidos a la inhibición de esta enzima.

A su vez los receptores beta se han subdividido en $beta_1$ y $beta_2$, (4). Ariens et al (5) sugieren que los efectos $beta_1$ son debidos preferentemente a la interacción del neurotransmisor norepinefrina con este receptor, mientras que para los receptores $beta_2$ la hormona epinefrina es más potente que el neurotransmisor norepinefrina.

En 1977 Berthelsen y Pettinger (6) subdividen a su vez a los receptores alfa en $alfa_1$ y $alfa_2$, sin embargo a diferencia de los

receptores beta adrenérgicos a los cuales se les considera isorreceptores, los receptores alfa actúan a través de diferentes sistemas de transducción. Fain y García-Sáinz en 1980 (7) proponen que los receptores alfa, involucran un recambio de fosfatidilinositol (PI) en la membrana plasmática y un cambio en la homeostasis del Ca^{2+} intracelular, mientras que los receptores $alfa_2$ actúan provocando una inhibición de la adenilato ciclasa a través de un mecanismo independiente de Ca^{2+}.

1.3 Receptores Adrenérgicos y Adenilato Ciclasa.

Como se dijo anteriormente tres de los receptores adrenérgicos son capaces de interactuar con la enzima adenilato ciclasa localizada en la membrana plasmática (fig.1.1).

En el sistema de la adenilato ciclasa la activación de los receptores beta adrenérgicos provoca la estimulación de la enzima, mientras que la activación de los receptores $alfa_2$ la inhiben.

Se sabe que dichos receptores y la adenilato ciclasa forman parte de un complejo integrado por tres entidades: el receptor, una proteína reguladora denominada proteína "N" la cual posee alta afinidad por nucleótidos de guanina (8,9) y la subunidad catalítica de la adenilato ciclasa; por consiguiente, la respuesta inmediata a la interacción hormona-receptor se inicia con la formación de un complejo oligomérico que controla la actividad de la unidad catalítica de la adenilato ciclasa. Recientemente se ha demostrado que la proteína "N" involucrada en la activación de la adenilato ciclasa es diferente de la que participa en la inhibición (8, 10-12). De acuerdo a la terminología propuesta

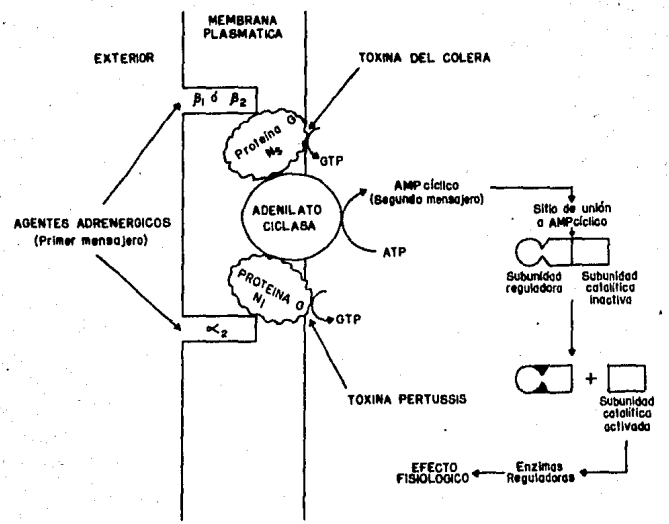

FIG.1.1 MODELO DE LOS COMPONENTES DEL SISTEMA DE TRANSDUCCION HORMONAL DE LA ADENILATO CICLASA. Ns, proteína reguladora (activación de la adenilato ciclasa); Ni, proteína reguladora (inhibición de la adenilato ciclasa).

por Rodbell en 1980 (8), estas proteínas se han denominado Ns a la que estimula a la adenilato ciclasa y Ni a la que la inhibe. Estas subunidades reguladoras tienen la capacidad de unir GTP y de esta manera cambiar la conformación de la adenilato ciclasa a la forma activa o inactiva respectivamente; posteriormente el GTP es hidrolizado por una GTPasa, convirtiendo a la adenilato ciclasa a la forma previa.

En el estudio de los mecanismos de transducción de receptores acoplados a la adenilato ciclasa, se han utilizado en forma muy importante dos toxinas: la toxina del cólera que interfiere selectivamente con el mecanismo de activación de la adenilato ciclasa, y la toxina pertussis que bloquea las respuestas inhibitorias de esta enzima.

La toxina del cólera es una proteína formada por dos entidades, de las cuales la subunidad A modifica a la proteína Ns la cual controla la actividad catalítica de la adenilato ciclasa, catalizando la transferencia de una unidad ADP-ribosa del NAD^+ a un residuo de arginina. Esta ADP ribosilación bloquea la actividad de la GTPasa, por lo tanto la proteína Ns no puede ser desactivada una vez que se estimula para incrementar los niveles de adenosina 3',5'-monofosfato cíclico, (AMPc). Este efecto es reproducido por análogos de AMPc y por análogos no hidrolizables de GTP, (fig.1.1).

Al igual que la toxina del cólera, la toxina pertussis, producida por la Bordetella pertussis y su relación con este sistema, ha recibido un gran auge en los últimos años. En 1981 García-Sáinz (14) demuestra que la vacuna pertussis produce una disminución en la sensibilidad de las catecolaminas alfa$_2$-adre-

nérgicas, y que este efecto no es exclusivo para estos agentes sino que también se observa para prostaglandinas, adenosina y ac. nicotínico proponiendo que un componente de la vacuna pertussis bloquea la transferencia de la información inhibitoria del receptor a la adenilato ciclasa. Este componente es la toxina pertussis que ha sido utilizado como arma para el estudio de la regulación de la ciclasa por diversos grupos (15-20).

1.4 Receptores alfa$_1$-adrenérgicos.

A diferencia del adelanto que se tiene en el conocimiento del mecanismo de transducción de los receptores beta y alfa$_2$ adrenérgicos y del sistema de la adenilato ciclasa, en general, el mecanismo de trasducción de los receptores alfa$_1$-adrenérgicos ha tenido poco progreso y no es sino en los últimos 5 años cuando se ha tenido cierto avance en este tema.

En 1980 Fain y García-Sáinz (7) propusieron que las acciones alfa$_1$ adrenérgicas eran mediadas por un recambio en el metabolismo del fosfatidilinositol y que este fenómeno modificaba de manera significativa la homeostasis de los iones Ca^{2+}, y que estos iones *per se* jugaban un papel muy importante.

Varios trabajos demostraban que los efectos fisiológicos de ciertas hormonas en hígado, incluyendo las catecolaminas alfa$_1$-adrenérgicas producían alteraciones en los flujos de Ca^{2+}. Vasopresina, angiotensina II y los agentes alfa$_1$ -adrenérgicos regulan a la glucógeno fosforilasa a través de mecanismos que involucran la elevación del Ca^{2+} intracelular (21-23). Esta movilización de los iones Ca^{2+} en el hígado de rata inducido por hormonas dependientes de Ca^{2+}, se explica no solo en términos de la inhi-

bición de la Ca^{2+}-ATPasa localizada en la membrana, sino que el eflujo de Ca^{2+} parece ser debido a la movilización de este catión de organelos intracelulares, lo cual se ve reflejado en incrementos del Ca^{2+} citosólico. Aún no está claro cual es el sitio intracelular que funciona como depósito donador de Ca^{2+} y el mecanismo por el cual se efectúa el proceso de la elevación del Ca^{2+} citosólico. Varios estudios presentan evidencias de que este depósito de Ca^{2+} intracelular puede ser la mitocondria (24,25), otros autores sugieren que se trata de depósitos ajenos a la mitocondria como el retículo endoplásmico, (26,27) o ambos (28). Con excepción de la membrana plasmática, el sitio de donde se movilizará calcio requeriría de la intervención de una molécula que actuara como segundo mensajero.

Michell (29) propuso en 1981 que el recambio del fosfatidil inositol (PI) en la membrana plasmática era la respuesta primaria a la interacción de las hormonas dependientes de Ca^{2+} con su receptor, y que esto era lo que provocaba los flujos de Ca^{2+}. Michell demostró en diferentes tejidos que la estimulación del recambio del PI era una respuesta constante de agentes como vasopresina, angiotensina II y agentes alfa$_1$-adrenérgicos y que este efecto no se asociaba a agentes que involucran aumentos de AMPc. Se ha postulado que la interacción de estos agonistas con su receptor activa a una fosfolipasa lo cual resulta en un incremento en la degradación del PI en la membrana plasmática convirtiéndolo en 1,2-diacilglicerol y mio-inositol-1,2-fosfato cíclico. La fosforilación del grupo hidroxilo libre del diacilglicerol por ATP produce ác. fosfatídico (PA), el cual es resintetizado a PI por una CDP-diacil-glicerol inositol transferasa en retículo

endoplásmico. Hasta esta fecha no se sabía cual de los productos de este metabolismo, (el PI, el fosfatidilinositol 4,5-bifosfato (PIP_2), el fosfatidil- inositol- fosfato, o el inositol 1,4,5,trifosfato (IP_3),) producía la movilización del Ca^{2+}. Los candidatos para actuar como segundos mensajeros eran el ác. fosfatídico, el mio-inositol fosfato(s) o una proteína cinasa Ca^{2+}-dependiente, activada por diacilglicerol llamada proteína cinasa C (30,31).

A pesar de estos datos el mecanismo a través del cual agentes como la vasopresina, la epinefrina y la angiotensina II ejercen su mecanismo de acción intracelular permanecía obscuro; se sabía que estos agentes estimulaban el recambio del PI y elevación de mio-inositol en hepatocitos aislados. Sin embargo estos cambios no eran significativos antes de 2 minutos. Era concluyente que estos fenómenos eran muy lentos comparados con los incrementos en la concentración de Ca^{2+} en el citosol y la activación de la glucógeno fosforilasa causada por estos agentes, los cuales ocurren alrededor de 2 segundos.

En 1981 Kirk et al (32) y Michell et al (33) postularon que la degradación del PIP_2 pudiera ser el factor primario en este mecanismo de acción.

En 1985 Berridge e Irvine (34) demuestran en la glándula salival de la mosca que la hidrólisis del PIP_2 en IP_3 y 1,2 diacilglicerol ocurría en 3 segundos, estos datos eran consistentes con la idea de que el IP_3 pudiera funcionar como segundo mensajero movilizando Ca^{2+} intracelular, estos autores también demostraron que la adición de IP_3 en el rango micromolar inducía

la movilización de calcio de la membrana interna del retículo endoplásmico, este efecto parecía ser específico de IP_3 ya que no se obtenía respuesta al agregar IP_2, IP o inositol 1,2-cíclico fosfato.

Otro aspecto muy importante de este sistema es la generación de diacilglicerol (DG) al mismo tiempo que el IP_3. El DG por su naturaleza liposoluble permanece en la bicapa lipídica de la membrana plasmática antes de ser metabolizado a ác. fosfatídico por una diacilglicerido cinasa o en algunos casos a ác. araquidónico por una diacilglicerido lipasa. El descubrimiento de Nishizuka (31) de una proteína cinasa activada por diacilglicerol, dependiente de Ca^{2+} y fosfolípidos, llamada proteína cinasa C, sugiere que el DG per se puede tener una función importante en este mecanismo de transducción hormonal.

Los ésteres de forbol son una serie de agentes tumorigénicos, el más potente de ellos es el 4 beta-forbol-12-miristato-13-acetato (PMA), el cual es capaz de activar directamente a la proteína cinasa C. Hay algunos reportes que demuestran sinergismo entre ionóforos de calcio y PMA en plaquetas (31,36), páncreas (52) y otros sistemas secretores (31,53), sin embargo recientemente se ha reportado inhibición de la respuesta alfa$_1$-adrenérgica por PMA (54,55). Estos datos sugieren que la proteína cinasa C puede estar involucrada en procesos de desensibilización o modificación en el número de receptores de la hormona (fenómeno conocido como down-regulation).

El PMA estimula la fosforilación de la proteína ribosomal S_6 (Mr 32 000) en una línea celular (57). El papel de la subunidad ribosomal 40 S en el proceso de iniciación en la síntesis de

proteínas sugieren un papel regulador de la proteína cinasa C en este proceso (58). Por otro lado se ha reportado que el PMA causa inactivación de la glucógeno sintasa en hepatocitos (60), también se ha demostrado fosforilación de esta enzima inducida por PMA tanto en hígado como en músculo (60).

Es interesante recalcar la función de las dos moléculas generadas en la hidrólisis del PIP_2, ya que mientras el DG activa a la proteína cinasa C el IP_3 mobiliza Ca^{2+} el cual también activa una serie de proteinas cinasas contribuyendo ambos efectos a la respuesta final de la cascada en la señal hormonal (fig. 1.2).

Resulta interesante comparar este sistema con el de la adenilato ciclasa, ya que podemos encontrar algunas similitudes: las moléculas que actúan como segundos mensajeros son generadas a partir de precursores fosforilados localizados en la membrana plasmática, los niveles intracelulares de estos mensajeros son determinados por balance de masa entre las velocidades de formación y degradación de estos compuestos, en ambos mecanismos existen sistemas que metabolizan rápidamente a las moléculas que actúan como segundos mensajeros (AMPc, IP_3 y DG), inclusive en los últimos tres años se ha propuesto (37-38) que los nucleótidos de guanina pueden jugar un papel importante en la regulación de los receptores $alfa_1$-adrenérgicos, probablemente actuando en una proteína similar a Ns, sin embargo aun se necesita profundizar más sobre este tema (fig.1.3).

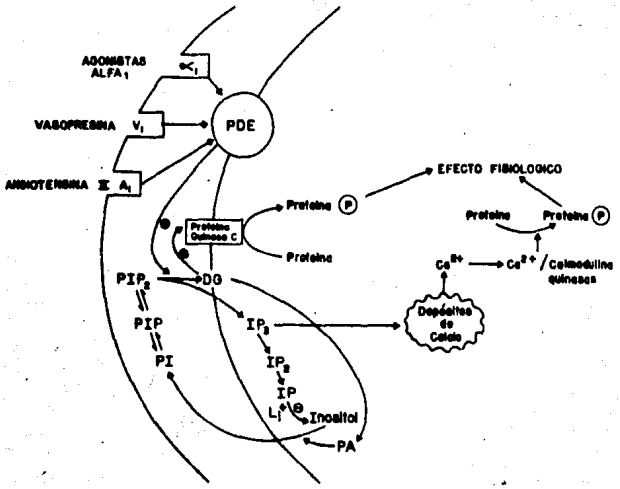

FIG.1.2 MODELO DE ACCION HORMONAL PARA LOS AGENTES ALFA$_1$-ADRENERGICOS. PDE, fofodiesterasa; PIP_2, fosfatidil inositol 4,5-bifosfato; IP_3, inositol 1,4,5-trifosfato; DG, 1,2-diacilglicerol; PA, ác. fosfatídico.

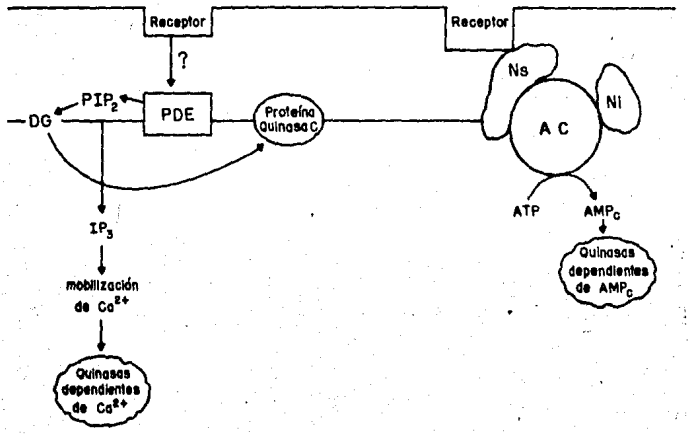

FIG. 1.3 COMPARACION ENTRE LOS MECANISMOS DE ACCION HORMONAL A NIVEL DE LA MEMBRANA PLASMATICA. Otras indicaciones son iguales que en los pies de las figuras 1.1 y 1.2.

2. POSIBLE EXISTENCIA DE DOS MECANISMOS DE ACCION PARA EL RECEPTOR ALFA$_1$-ADRENERGICO.

2.1 Antecedentes.

Previamente se ha propuesto que las hormonas que movilizan iones calcio como los agentes alfa$_1$-adrenérgicos, vasopresina y angiotensina II, provocan la hidrólisis del PIP_2 en la membrana plasmática mediante la activación de una fosfolipasa C, obteniéndose como productos de esta hidrólisis el IP_3 y el DG. Se cree que el IP_3 sea el agente que moviliza el calcio de depósitos intracelulares, preferentemente el del retículo endoplásmico. El DG activa a la proteína cinasa C localizada en la membrana plasmática. Esta serie de eventos parecen ser los que desencadenan la cascada de la señal hormonal dando como efecto final la alteración de rutas metabólicas ya establecidas. Sin embargo hay una serie de evidencias que sugieren diferencias en el mecanismo de acción de los agentes alfa$_1$-adrenérgicos en comparación con el de otras hormonas como vasopresina y angiotensina II (39-50). A continuación se describen algunas de ellas.

a) Los efectos metabólicos producidos por agentes alfa$_1$-adrenérgicos son claramente observados en hepatocitos incubados en ausencia de calcio extracelular o en hepatocitos depletados de calcio, pero no así los efectos debidos a los péptidos presores vasopresina y angiotensina II, (39,48).

b) El hipotiroidismo disminuye significativamente los efectos metabólicos de vasopresina y angiotensina II pero no los de las aminas alfa$_1$-adrenérgicas, (40,42).

c) La insulina disminuye la glucogenólisis estimulada por epinefrina pero no la inducida por los péptidos presores (43-

45,48).

d) La acción inhibitoria de la insulina en la respuesta alfa$_1$-adrenérgica es mayor en hepatocitos depletados de calcio y en hepatocitos de animales hipotiroideos que en células control. (43,44,48).

e) Por otro lado se sabe que los glucocorticoides al igual que las hormonas tiroideas tienen el llamado "efecto permisivo" (que es la capacidad que tienen algunas hormonas de alterar la respuesta celular a otras) sobre la respuesta alfa$_1$-adrenérgica, (37,51) ya que al estudiar la respuesta adrenérgica en animales adrenalectomizados se ve un aumento de la respuesta beta acompañada de una disminución tanto en la sensibilidad alfa$_1$- adrenérgica como en la movilización de calcio intracelular.

f) Lardy et al (61,62) demostraron que la gluconeogénesis a partir de sustratos que involucran la acción de la fosfoenolpiruvato carboxicinasa como son el lactato o el piruvato es estimulada por catecolaminas, vasopresina y angiotensina II y que la respuesta es dependiente de calcio; sin embargo cuando se utiliza como sustrato a la dihidroxiacetona, los péptidos presores no tienen ningun efecto en la estimulación de esta vía metabólica, mientras que norepinefrina si la estimula significativamente.

g) Para finales de 1984 Huerta-Bahena y García-Sáinz (64) demostraron que la cicloheximida a la cual habían caracterizado previamente como un agente alfa$_1$-adrenérgico (63), actuaba a través de un mecanismo dependiente de calcio extracelular encontrándose de esta manera un parámetro adicional, para sugerir dos mecanismos de acción alfa$_1$-adrenérgica. Esta serie

de antecedentes llevó a proponer la siguiente hipótesis de trabajo.

2.2 Hipótesis.

El receptor alfa$_1$ -adrenérgico está acoplado a dos mecanismos de transducción, uno de esos mecanismos está asociado al recambio de PIP_2 y generación de IP_3 y DG en la membrana plasmática, es dependiente de la concentración de calcio extracelular, insensible a una inhibición por insulina y modulado por hormonas tiroideas, este mecanismo es compartido con vasopresina y angiotensina II, mientras que el otro mecanismo es independiente de la concentración de calcio extracelular, sensible a una inhibición por insulina y modulado por glucocorticoides, (no compartido con los péptidos presores). Lo anterior se resume en la figura 2.1. El objetivo general de esta tesis es estudiar el mecanismo de acción de los agentes alfa$_1$-adrenérgicos.

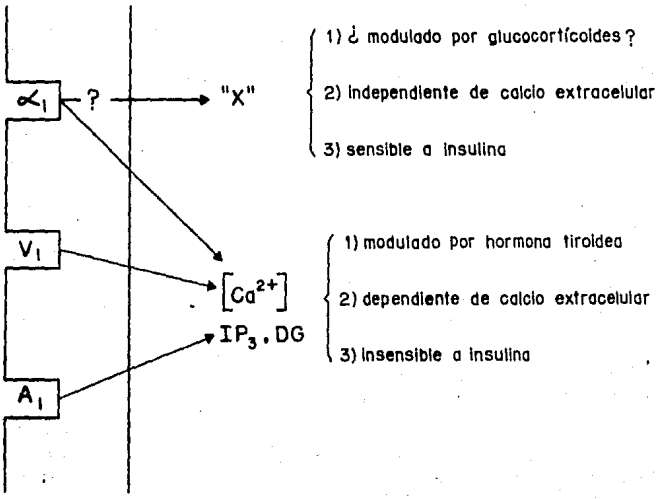

FIG. 2.1 ESQUEMA DE LOS DOS POSIBLES MECANISMOS DE ACCION ALFA$_1$-ADRENERGICA. IP$_3$, inositol 1,4,5, trifosfato; DG, 1,2-diacilglicerol.

II. MATERIALES Y METODOS

La parte correspondiente a materiales y métodos se da en los trabajos respectivos.

I I I. R E S U L T A D O S

Los resultados obtenidos en la realización de esta tesis han sido publicados en los siguientes trabajos:

1. Hernández-Sotomayor S.M.T. and García-Sáinz J.A., (1984). Adrenergic regulation of ureogenesis in hepatocytes from adrenalectomized rats. Possible involvement of two pathways of signal transduction in alpha$_1$-adrenergic action . FEBS Lett.166, 385-388.

2. García-Sáinz J. A. and Hernández-Sotomayor S. M.T., (1985). Adrenergic regulation of gluconeogenesis. Possible involvement of two mechanisms of signal transduction in alpha$_1$-adrenergic action. Proc. Natl. Acad. Sci. U.S.A., 82, 6727-6730.

3. García-Sáinz J. A., Tussié-Luna M. I. and Hernández-Sotomayor S.M.T. (1985). Alpha$_1$ adrenergic desensitization induced by phorbol esters, vasopressin and angiotensisn II in rat hepatocytes. FEBS Lett. Sometido.

4. García-Sáinz J. A., Hernández-Sotomayor S.M.T. and Tussié-Luna M. I. (1985) Homologous and heterologous desensitization af one of the pathways of the alpha$_1$-adrenergic action. Effects of epinephrine, vasopressin, angiotensin II and phorbol 12-myristate 13-acetate. Biochem. Biophys. Acta. Sometido.

RESUMEN DE RESULTADOS

Trabajo 1. El objetivo de este trabajo fue determinar el efecto permisivo de los glucocorticoides en la respuesta hepática alfa$_1$-adrenérgica, obteniéndose los siguientes resultados:

a) A diferencia de los animales controles en los cuales la respuesta adrenérgica es predominantemente del tipo alfa$_1$, en animales adrenalectomizados se encuentran involucrados los receptores alfa$_1$ y beta adrenérgicos.

b) La respuesta alfa$_1$ - adrenérgica en animales adrenalectomizados es dependiente de la concentración de calcio extracelular.

c) La respuesta alfa$_1$ -adrenérgica en animales adrenalectomizados es insensible a una inhibición por insulina.

d) La administración de dexametasona a animales adrenalectomizados revierte la independencia de calcio extracelular para la respuesta alfa$_1$- adrenérgica.

Trabajo 2. El objetivo de este trabajo fue someter una vía metabólica al esquema general de nuestra hipótesis de la acción alfa$_1$-adrenérgica, escogiéndose como modelo la gluconeogénesis a partir de dihidroxiacetona (DHA) y lactato, obteniéndose los siguientes resultados.

a) La gluconeogénesis a partir de ambos sustratos, DHA o lactato, es estimulada significativamente por agentes alfa$_1$-adrenérgicos en presencia o en ausencia de calcio extracelular.

b) La gluconeogénesis a partir de lactato es estimulada por vasopresina y angiotensina II solo en presencia de calcio

extracelular.

c) La gluconeogénesis a partir de DHA no es estimulada por vasopresina ni por angiotensina II aun en presencia de calcio extracelular.

d) La gluconeogénesis a partir de DHA es estimulada por agentes alfa$_1$-adrenérgicos en hepatocitos de animales hipotiroideos pero no en hepatocitos de animales adrenalectomizados.

e) La gluconeogénesis a partir de lactato estimulada por agentes alfa$_1$-adrenérgicos es sensible a una inhibición por insulina solo en ausencia de calcio extracelular.

f) La gluconeogénesis a partir de DHA estimulada por epinefrina es altamente sensible a una inhibición por insulina tanto en ausencia como en presencia de calcio extracelular.

h) La gluconeogénesis es estimulada por cicloheximida solo cuando el sustrato es lactato.

Trabajo 3. El objetivo de este trabajo fue demostrar que hormonas que activan a la proteína cinasa C como la vasopresina y la angiotensina II eran capaces de inhibir al igual que los ésteres de forbol, la respuesta alfa$_1$-adrenérgica, en esta parte del estudio se obtuvieron los siguientes resultados.

a) La gluconeogénesis a partir de DHA estimulada por epinefrina en presencia de propranolol 1 uM es inhibida de una manera dependiente de la dosis, por vasoprenina, angiotensina II y PMA encontrándose el siguiente orden de potencia: vasopresina > angiotensina II = PMA.

b) La ureogénesis estimulada por epinefrina en presencia

de propranolol 10 uM en hepatocitos de animales normales incubados sin calcio en presencia de EGTA 25 uM es inhibida por los tres agentes en el siguiente orden de potencia: vasopresina>= PMA> angiotensina II.

c) La ureogénesis estimulada por epinefrina en presencia de propranolol 10 uM en hepatocitos de animales hipotiroideos también es inhibida por los tres agentes con el siguiente orden de potencia: vasopresina=PMA > angiotensina II.

Trabajo 4. El objetivo de este trabajo fue tratar de caracterizar la desensibilización alfa$_1$-adrenérgica causada por agentes que activan a la proteína cinasa C, obteniéndose los siguientes resultados:

a) La desensibilización alfa$_1$-adrenérgica inducida por vasopresina, angiotensina II y PMA es reversible solo en presencia de calcio extracelular.

b) La desensibilización homóloga alfa$_1$-adrenérgica causada por epinefrina prácticamente no es reversible aún en presencia de calcio extracelular.

c) La desensibilización causada por estos agentes es selectiva para la respuesta alfa$_1$-adrenérgica, ya que al probar los efectos metabólicos de otra hormona como es el glucagon prácticamente ésta no se ve afectada.

d) La estimulación alfa$_1$-adrenérgica para el recambio de fosfatidilinositol se recupera totalmente tanto en presencia como en ausencia de calcio, excepto cuando el agente utilizado para la desensibilización es la epinefrina.

e) El recambio de fosfatidilinositol inducido por vasopresina no es afectado por ninguno de los agentes probados.

f) En células de animales hipotiroideos hay una escasa reversión de la desensibilización inducida por vasopresina, angiotensina II, PMA y epinefrina (sistema equivalente a una incubación en ausencia de Ca^{2+}), mientras que en células de animales adrenalectomizados la respuesta alfa$_1$-adrenérgica se recupera totalmente con todos los agentes y sólo parcialmente con epinefrina (sistema equivalente a una incubación con calcio).

I V. D I S C U S I O N

Existen una serie de evidencias que sugieren diferencias en el mecanismo de acción de hormonas como vasopresina, angiotensina II y las aminas alfa$_1$-adrenérgicas (39-48). En la hipótesis de trabajo de esta tesis se propone la existencia de dos mecanismos de transducción para los agentes alfa$_1$-adrenérgicos (fig. 2.1). En el presente trabajo se obtuvieron datos consistentes con esta hipótesis; se encontró que efectivamente los glucocorticoides son capaces de modular el mecanismo de acción alfa$_1$-adrenérgico independiente de calcio extracelular, sensible a insulina (trabajo 1).

Por otro lado se encontró que existe una vía metabólica, ya no en un estado patológico como lo es la adrenalectomía o el hipotiroidismo, sino un parámetro en el animal normal el cual es regulado por el mecanismo alfa$_1$-adrenérgico independiente de Ca^{2+}: la gluconeogénesis a partir de DHA (trabajo 2).

Los ésteres de forbol son una serie de compuestos tumorigénicos que tienen diversas acciones en una gran variedad de tejidos, muchas de las acciones de estos compuestos se atribuyen a la activación de la proteína cinasa C (30,65), como ya se ha visto, se sugiere que esta enzima este involucrada en el mecanismo de acción de algunas hormonas como vasopresina y agentes alfa$_1$-adrenérgicos.

Se sabe que estos agentes son capaces de alterar la afinidad por sus agonistas de una variedad de receptores (66,67) aunque no se concoce aún el mecanismo a través del cual los ésteres de

forbol provocan estos efectos, hay evidencias sobre la fosforilación de algunos receptores producida por los ésteres de forbol (61) la inhibición de la respuesta alfa$_1$-adrenérgica producida por PMA pudiera estar mediada por la activación de la proteína cinasa C que a su vez sería responsable de la fosforilación del receptor.

Existen varios sistemas que sugieren que la proteína cinasa C puede funcionar como un elemento bidireccional (69) en la regulación del metabolismo intracelular ya sea que pueda activar o inhibir los efectos del mismo agonista, sin embargo aun no está claro el efecto fisiológico de este proceso. Algunas enzimas del metabolismo de los lípidos del inositol parecen ser reguladas por proteína cinasa C, en una gran variedad de tejidos la resíntesis de los polifosfoinosítidos es estimulada por proteína cinasa C (70).

En el tercer trabajo que constituye esta tesis nuestros resultados muestran la inhibición del mecanismo no convencional de la respuesta alfa$_1$-adrenérgica por vasopresina, angiotensina II y ésteres de forbol, de estos resultados podemos enfatizar el siguiente punto: esta inhibición se lleva a cabo a dosis relativamente bajas de estos agentes, sin embargo a dosis fisiológicas normales no ocurre la inhibición, es decir a concentraciones en las que se presentan vasopresina y angiotensina II en condiciones normales en el organismo no actúan como antagonistas alfa$_1$-adrenérgicos, pero en condiciones en las cuales hay una elevación de estos agentes además de ejercer sus efectos fisiológicos (como vasopresores, estimuladores del metabolismo, etc.), pueden inhibir los efectos fisiológicos de la epinefrina, (por ejemplo en el

shock hipovolémico).

A lo largo de esta tesis se ha mencionado la posible existencia de dos "mecanismos de transducción" para los agentes alfa$_1$-adrenérgicos, sin embargo no podemos descartar la posibilidad de que se trate de dos receptores cada uno acoplado a un mecanismo de acción diferente.

Los resultados presentados en el cuarto trabajo apoyan este hecho, ya que sólo es reversible la desensibilización inducida al mecanismo dependiente de calcio (incubaciones en presencia de calcio y animales adrenalectomizados), mientras que la desensibilización del mecanismo alternativo o independiente de calcio no lo es. Es posible que la proteína cinasa C provoque una desensibilización transitoria del receptor acoplado al sistema convencional, mientras que en el otro caso se provoque un fenómeno más duradero.

Si bien nuestros datos son congruentes con la hipótesis de trabajo aún queda una pregunta por responder ¿cuál es la molécula que actúa como segundo mensajero en este sistema alternativo de transducción alfa$_1$-adrenérgico? Algunos grupos han propuesto que esta entidad podría ser el AMPc. Chan y Exton (71,77) han detectado aumento en los niveles de AMPc en hepatocitos depletados de calcio. Sin embargo cuando utilizamos como modelo a los animales hipotiroideos donde suponemos actúa el mecanismo alternativo a IP_3 y Ca^{++}, no se detectó estimulación significativa del AMPc inducida por epinefrina en presencia de propranolol (41). Por otro lado Lardy et al (61) reportaron que el atractilósido inhibe la gluconeogénesis a partir de DHA producido por AMPc o glucagon

pero no la estimulada por epinefrina, estos datos no apoyan el hecho de que el segundo mensajero del mecanismo altervativo pudiera ser el AMPc.

En resumen nuestros datos apoyan la hipótesis que sugiere la existencia de dos mecanismos de transducción para los agentes alfa$_1$-adrenérgicos.

a) Uno "convencional", común a los péptidos presores que involucra al IP_3 y al DG como segundos mensajeros provocando la mobilización de Ca^{2+} intracelular modulado por hormonas tiroideas, dependiente de calcio extracelular, insensible a una inhibición por insulina, cuyos efectos son reproducidos por cicloheximida, y

b) Una vía "alternativa" cuyo segundo mensajero no se conoce modulado por glucocorticoides, independiente de calcio extracelular, sensible a una inhibición por insulina, sus efectos no son reproducidos por cicloheximida.

Es importante mencionar que la evidencia es bastante amplia (39-43, 64, y la presente tesis) pero circunstancial; mientras no conozcamos el segundo mensajero no tendremos una evidencia definitiva. Confiamos en que la amplia caracterización que hemos hecho permita lograr este objetivo en un futuro no muy lejano.

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Adrenergic regulation of ureogenesis in hepatocytes from adrenalectomized rats

Possible involvement of two pathways of signal transduction in α_1-adrenergic action

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In hepatocytes from control rats, the ureogenic action of epinephrine is mainly mediated through α_1-adrenoceptors and the effect is independent of the presence of extracellular calcium. In hepatocytes from adrenalectomized rats, both α_1- and β-adrenoceptors are involved in the action of epinephrine. Furthermore, the α_1-adrenergic-mediated stimulation of ureogenesis in these cells is dependent on the presence of extracellular calcium. Our results indicate that glucocorticoids modulate the calcium dependency of α_1-adrenergic effects and are consistent with our suggestion that two pathways are involved in the transduction of the α_1-adrenergic signal.

α_1-Adrenergic receptor *Ureogenesis* *β-Adrenergic receptor* *Adrenalectomy*

1. INTRODUCTION

Thyroid hormones and glucocorticoids are known modulators of the actions of other hormones, including catecholamines (permissive effects). We have previously shown that in hepatocytes from hypothyroid rats the metabolic actions of vasopressin and angiotensin II are markedly diminished whereas the effects of α_1-adrenergic amines are not [1,2] but become extremely sensitive to the antagonistic action of insulin (submitted). The data suggested that the action of α_1-adrenergic agents may involve two pathways: one, shared with vasopressin and angiotensin II, which is dependent on extracellular calcium, probably involves phosphatidylinositol turnover and is insensitive to insulin; and the other, which is independent of extracellular calcium and sensitive to insulin [1,2]. We have therefore studied the effect of glucocorticoid deficiency (adrenalectomy) on hormonal regulation of ureogenesis. It was observed that in hepatocytes from adrenalectomized rats α_1-adrenergic stimulation of ureogenesis became dependent on the presence of extracellular calcium and was not affected by insulin.

2. MATERIALS AND METHODS

The sources of materials were the same as in [1–3]. Female Wistar rats (~200 g) fed ad libitum were used. Bilateral adrenalectomy was performed by a dorsal approach; adrenalectomized animals were given 0.85% NaCl to drink and were used 5–8 days after operation. Hepatocytes were isolated and incubated under conditions to study

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ureogenesis as in [1–3]. Urea was determined as in [4]. The dependency of extracellular calcium of hormonal effects was determined by adding EGTA (final concentration 2.5 mM, adjusted to pH 7.4) to the incubation buffer which contained 1.2 mM $CaCl_2$.

3. RESULTS

Epinephrine stimulated ureogenesis to the same extent in hepatocytes from control or adrenalectomized animals (fig.1). In contrast, isoproterenol was much more effective in hepatocytes from adrenalectomized animals than in control cells (fig.1) suggesting involvement of β-adrenoceptors in the action of epinephrine in cells from adrenalectomized animals. Studies with adrenergic antagonist were consistent with the findings, i.e., the effect of epinephrine was abolished by prazosin in hepatocytes from control animals, indicating that the action of the hormone is mediated mainly through α_1-adrenoceptors [3] (fig.2) whereas in hepatocytes from adrenalectomized rats only the addition of both prazosin and propranolol blocked

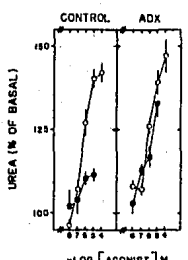

Fig.1. Effect of epinephrine or isoproterenol on ureogenesis in hepatocytes from control or adrenalectomized rats. Hepatocytes from control or adrenalectomized (ADX) rats were incubated in the presence of different concentrations of epinephrine (○) or isoproterenol (■). Means (± SE) are plotted of duplicate incubations from 4–8 cell preparations. Results are expressed as percentage of basal urea production during 60 min, which was 22 ± 2 and 25 ± 2 nmol/mg cell wet wt in cells from control and adrenalectomized rats, respectively.

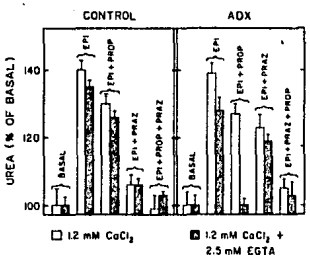

Fig.2. Effect of adrenergic antagonist on the stimulation of ureogenesis by epinephrine and role of extracellular calcium. Hepatocytes from control or adrenalectomized (ADX) rats were incubated for 60 min in medium containing 1.2 mM $CaCl_2$ (open bars) or 1.2 mM $CaCl_2$ plus 2.5 mM EGTA (hatched bars) in the presence of 10^{-5} M epinephrine (EPI); 10^{-5} M epinephrine + 10^{-5} M propranolol (EPI + PROP); 10^{-5} M epinephrine + 10^{-5} M prazosin (EPI + PRAZ) or 10^{-5} M epinephrine + 10^{-5} M propranolol + 10^{-5} M prazosin (EPI + PROP + PRAZ). Results are the means (± SE) of duplicate incubations from 4–8 cell preparations and are expressed as percentage of basal urea synthesis. Basal urea production in the presence of 1.2 mM $CaCl_2$ is given in fig.1; urea production in the presence of 1.2 mM $CaCl_2$ + 2.5 mM EGTA was 20 ± 1 and 29 ± 3 nmol/mg cells wet wt in cells from control and adrenalectomized rats, respectively.

the effect of epinephrine, showing that in these cells both α_1- and β-adrenoceptors are involved in the action of the amine (fig.2).

To evaluate further the α_1-adrenergic sensitivity of the cells, experiments were performed in the presence (1.2 mM $CaCl_2$) or absence (1.2 mM $CaCl_2$ plus 2.5 mM EGTA) of extracellular calcium (fig.3). In agreement with our previous studies [5], the ureogenic effect of epinephrine + propranolol is clearly observed in both the presence or absence of extracellular calcium in cells from control animals. Interestingly, in cells from adrenalectomized rats, the effect of epinephrine plus propranolol was abolished in the absence of calcium (fig.2,3). Some effect of epinephrine (in the presence of propranolol) was observed in cells incubated in the absence of calcium; however, it

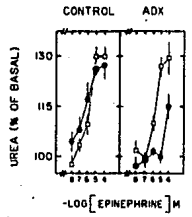

Fig.3. Role of extracellular calcium in the α_1-adrenergic-mediated stimulation of ureogenesis. Hepatocytes from control or adrenalectomized (ADX) rats were incubated for 60 min in the presence of 10^{-5} M propranolol and different concentrations of epinephrine in buffer containing 1.2 mM $CaCl_2$ (○) or 1.2 mM $CaCl_2$ + 2.5 mM EGTA (●). Results are the mean (± SE) of 4–8 cell preparations and are expressed as percentage of basal urea production. Basal rates of urea production are given in the legends to fig.1,2.

was observed at a very high concentration of epinephrine (10^{-4} M) at which incomplete β-adrenergic blockade by propranolol is very likely to occur. The absence of effect in medium containing EGTA was not due to general cell damage since clear effects of epinephrine alone or of epinephrine + prazosin were observed (fig.2). Vasopressin and angiotensin II stimulate ureogenesis in the presence of calcium in cells from either control or adrenalectomized rats (not shown).

Administration of dexamethasone to adrenalectomized rats (500 μg 48 and 24 h before the experiment was performed) restored the independence of extracellular calcium of the α_1-adrenergic stimulation of ureogenesis (116 ± 2% of basal level in the presence of 10^{-5} M epinephrine, 10^{-5} M propranolol and 2.5 mM EGTA; and 125 ± 2% of basal level in the presence of 10^{-4} M epinephrine + 10^{-5} M propranolol and 2.5 mM EGTA; means ± SE of 6 experiments). Furthermore, the effect of β-adrenergic agonists was significantly reduced in these cells as compared to that in cells from adrenalectomized rats not treated with the glucocorticoid.

We have previously shown that in cells from hypothyroid rats the α_1-adrenergic stimulation of ureogenesis is markedly diminished by insulin (submitted). In cells from adrenalectomized animals insulin was not able to antagonize significantly the effect of 10^{-5} M epinephrine plus 10^{-5} M propranolol (127 ± 3% of basal level in the absence of insulin as compared to 124 ± 2% in the presence of 10^{-9} M insulin; means ± SE of 8 experiments in each case).

4. DISCUSSION

Our findings here indicate that in hepatocytes from adrenalectomized rats β-adrenergic receptors play a significant role in the actions of epinephrine. The data are consistent with the observation by other authors that in liver from adrenalectomized animals the β-adrenergic-mediated activation of adenylate cyclase by epinephrine is enhanced [6,7]. This action seems to be related to an increased number of β-adrenoceptors in liver plasma membranes of adrenalectomized rats [8,9]. Our contribution in this respect is to show the involvement of β-adrenoceptors under this condition in a specific pathway: ureogenesis.

More relevant are our findings on α_1-adrenergic action. Previous studies have shown that the number of α_1-adrenoceptors in liver plasma membrane is not changed by adrenalectomy [10–12]. However, α_1-adrenergic effects are diminished in hepatocytes from adrenalectomized animals [10]. It has been observed that calcium depletion abolishes the effect of phenylephrine on phosphorylase in cells from adrenalectomized rats but not in control hepatocytes [10]. We confirmed this finding for ureogenesis and interpreted the data as suggesting that in hepatocytes from glucocorticoid-deficient rats epinephrine action proceeds mainly through the pathway shared with vasopressin and angiotensin II (dependency on extracellular calcium, insensitivity to insulin).

The effect of guanine nucleotides on the affinity state of α_1-adrenoceptors for agonists has recently been a matter of dispute [11–13]. It has been shown that such an effect of guanine nucleotides on α_1-adrenoceptors is modulated by glucocorticoids [13], i.e., the effect of guanine nucleotides is not observed in membranes from adrenalectomized rats but present in membranes from control animals and from adrenalectomized rats treated with glucocorticoids [13]. The

physiological role of guanine nucleotides (or a guanine nucleotide binding protein) in the process of signal transduction for α_1-adrenergic amines is far from clear. However, it is tempting to speculate that a relationship between the above-mentioned findings and our data may exist.

In summary, our data show that in hepatocytes from adrenalectomized rats the ureogenic effect of epinephrine is mediated by α_1- and β-adrenoceptors. Furthermore, in these cells the α_1-adrenergic action is dependent on the presence of extracellular calcium. Our data are consistent with the hypothesis that two pathways are involved in the α_1-adrenergic effects; one seems to be regulated by thyroid hormones whereas the other seems to be modulated by glucocorticoids.

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Biochemistry

Adrenergic regulation of gluconeogenesis: Possible involvement of two mechanisms of signal transduction in α_1-adrenergic action

(dihydroxyacetone/lactate)

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ABSTRACT We have previously suggested that the effects of α_1-adrenergic agents on hepatocyte metabolism involve two mechanisms: (*i*) a calcium-independent insulin-sensitive process that is modulated by glucocorticoids and (*ii*) a calcium-dependent insulin-insensitive process that is modulated by thyroid hormones. We have studied the effect of epinephrine (plus propranolol) on gluconeogenesis from lactate and dihydroxyacetone. It was observed that the adrenergic stimulation of gluconeogenesis from lactate seemed to occur through both mechanisms, whereas when the substrate was dihydroxyacetone the action took place exclusively through the calcium-independent insulin-sensitive process. This effect was absent in hepatocytes from adrenalectomized rats, suggesting that it is modulated by glucocorticoids.

It is well known that α_1-adrenergic agents, vasopressin, and angiotensin II stimulate glycogenolysis, gluconeogenesis, and ureogenesis in hepatocytes from normal rats through a cyclic AMP-independent mechanism associated with changes in the cytosolic concentration of calcium and with phosphoinositide turnover (1–4). Calcium, diacylglycerols, and inositol 1,4,5-trisphosphate are putative mediators of the action of these hormones (5–8).

During the last 4 years we (9–14) and others (15–22) have observed differences between the action of the vasopressor peptides and those due to α_1-adrenergic activation. These differences led us to propose the possible existence of two mechanisms of signal transduction for α_1-adrenergic action in the liver cell (9–14). Our hypothesis is schematically represented in Fig. 1 and is based mainly on the following findings: (*i*) metabolic effects due to α_1-adrenergic activation are clearly observed in cells incubated in the absence of extracellular calcium and even in calcium-depleted hepatocytes, whereas those of the vasopressor peptides are abolished (9, 14, 20); (*ii*) hypothyroidism markedly diminishes the metabolic effects of vasopressin and angiotensin II but not those due to α_1-adrenergic activation (11, 12); (*iii*) insulin reduces the stimulation of glycogenolysis due to α_1-adrenergic activation but not that produced by vasopressin or angiotensin II (13, 16, 17, 20); (*iv*) the inhibitory action of insulin on α_1-adrenergic actions is markedly magnified in calcium-depleted hepatocytes and in hepatocytes from hypothyroid rats (13, 16, 20); (*v*) in hepatocytes from adrenalectomized rats the metabolic effects due to α_1-adrenergic amines became dependent on the presence of extracellular calcium (14, 23)—i.e., α_1-adrenergic actions resemble those of vasopressin or angiotensin II; (*vi*) we have recently observed that cycloheximide, which stimulates hepatic metabolism through an α_1-adrenergic mechanism (24), mimics the actions of epinephrine in an insulin-insensitive calcium-dependent fashion and that the action of cycloheximide is observed in hepatocytes from control and adrenalectomized rats but not in cells from hypothyroid animals (25). Thus, in summary, our model suggests that α_1-adrenergic effects are mediated through two pathways: one of them also shared by vasopressin and angiotensin II, modulated by thyroid status, calcium-dependent, insulin-insensitive, and possibly involving phosphoinositide turnover and calcium in its mechanism of transduction; and another pathway, not shared with the vasopressor peptides, modulated by glucocorticoids, calcium-independent, insulin-sensitive, and mediated through unknown second messenger(s) (see Fig. 1).

Recently, Kneer and Lardy (15) reported that norepinephrine stimulates gluconeogenesis from dihydroxyacetone in the absence or presence of extracellular calcium. Interestingly, vasopressin and angiotensin II were unable to mimic the action of norepinephrine (15). These results prompted us to study comparatively the adrenergic regulation of gluconeogenesis from lactate and dihydroxyacetone in the light of our hypothesis, and the results are the subject of this manuscript.

MATERIALS AND METHODS

Materials. *l*-Epinephrine, *dl*-propranolol, glucose oxidase, peroxidase, arginine-vasopressin, angiotensin II, 6-*n*-propyl-2-thiouracil, lactate, dihydroxyacetone, cycloheximide, and EGTA, were obtained from Sigma. Bovine serum albumin (fraction V) and collagenase (type II) were obtained from Reheis (Kankakee, IL) and Worthington, respectively. Insulin was a generous gift from Eli Lilly.

Animals. Female Wistar rats (180–220 g) fasted 24 hr prior to the experiment were used. Hypothyroidism was induced by giving the rats water containing 0.030% 6-*n*-propyl-2-thiouracil for 30–40 days, and it was assessed by decreased weight gain, dryness of fur, and decreased blood levels of triiodothyronine (11, 12). Bilateral adrenalectomy was performed by a dorsal approach; adrenalectomized animals were given 0.85% NaCl to drink and were used 5–8 days after operation.

Hepatocyte Isolation and Metabolic Studies. Hepatocytes were isolated by the method of Berry and Friend (26) as modified by Tolbert *et al.* (2). Hepatocytes (~40 mg, wet weight) were incubated for 60 min at 37°C in a water-bath shaker in 1 ml of Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin at pH 7.4 under an atmosphere of 95% O_2/5% CO_2.

In all the experiments, the cells were incubated in the presence of 1 μM propranolol to block the β-adrenergic activity of the agents studied. Propranolol by itself did not affect the parameters studied. Glucose was determined in aliquots of the supernatant by the glucose oxidase-peroxidase method (27). Glucose synthesis from exogenous substrates (10 mM lactate or 2.5 mM dihydroxyacetone) has been corrected for synthesis from endogenous metabolites by subtracting the glucose production in the absence of sub-

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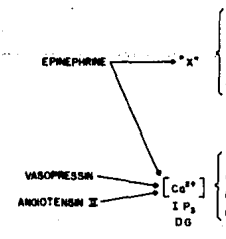

FIG. 1. Schematic representation of α_1-adrenergic action. IP_3, inositol 1,4,5-trisphosphate; DG, diacylglycerols.

strates from those values in the presence of substrate. All the data are the average of duplicate incubations of at least four different cell preparations.

RESULTS

Gluconeogenesis from Dihydroxyacetone and Lactate. Epinephrine, in the presence of 1 μM propranolol (to block its β-adrenergic activity) stimulated in a dose-dependent fashion the production of glucose from dihydroxyacetone or lactate (Fig. 2). The stimulation of glucose production from lactate was ≈60%, whereas that from dihydroxyacetone was only ≈20%.

To evaluate the role of extracellular calcium in the α_1-adrenergic-mediated stimulations of gluconeogenesis from these two substrates, cells were washed and incubated in buffer without $CaCl_2$ and containing 25 μM EGTA. Under these conditions, epinephrine was also clearly able to stimulate gluconeogenesis from both substrates (Fig. 2). Interestingly, the maximal stimulation of gluconeogenesis from dihydroxyacetone by epinephrine under these conditions was bigger (≈30%) than in the presence of calcium. In addition, the dose–response curve to epinephrine in the presence of lactate as substrate was shifted to the left (≈1 order of magnitude) in the absence of calcium as compared to the curve obtained in medium with calcium.

The effect of the vasopressor peptides, vasopressin and angiotensin II, on gluconeogenesis was studied and the results are presented in Fig. 3. In agreement with Kneer and Lardy (15), we observed that vasopressin and angiotensin II were ineffective in stimulating gluconeogenesis from dihydroxyacetone either in the absence or presence of extracellular calcium.

In contrast, both peptide hormones were able to stimulate gluconeogenesis from lactate in the presence of calcium; no effect of these peptides was observed in the absence of this cation (Fig. 3).

Studies with Hepatocytes from Hypothyroid Rats and Adrenalectomized Rats. Epinephrine in the presence of 1 μM propranolol stimulated gluconeogenesis from both dihydroxyacetone or lactate in hepatocytes from hypothyroid rats (Fig. 4). In contrast, in hepatocytes from adrenalectomized rats, epinephrine (plus 1 μM propranolol) was ineffective in stimulating gluconeogenesis from dihydroxyacetone but produced a clear dose-dependent stimulation of gluconeogenesis from lactate.

Effects of Insulin and Cycloheximide. The effect of insulin on the stimulations of gluconeogenesis from lactate or dihydroxyacetone by epinephrine is presented in Fig. 5. Insulin was without effect by itself. However, it abolished the stimulation of gluconeogenesis from dihydroxyacetone produced by epinephrine both in the presence or absence of calcium. In contrast, in the presence of lactate as substrate and in buffer containing calcium, insulin did not diminish the stimulation of gluconeogenesis produced by epinephrine. In the absence of calcium, insulin significantly diminished the stimulation of gluconeogenesis from lactate produced by epinephrine.

Cycloheximide, which seems to be a partial α_1-adrenergic

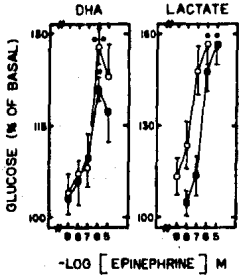

FIG. 2. Dose–response curves for the effect of epinephrine on gluconeogenesis from 2.5 mM dihydroxyacetone (DHA) or 10 mM lactate. Hepatocytes were incubated with substrates and agents for 60 min in the presence of 1.2 mM $CaCl_2$ (■) or in the absence of $CaCl_2$ and presence of 25 μM EGTA (○). Plotted are the means, and vertical lines represent the SEM of duplicate incubations from 4–8 cell preparations. Results are expressed as percentage of basal glucose synthesis from dihydroxyacetone, which was 18 ± 1 and 13 ± 1 nmol per mg of cells (wet weight) in the presence or absence of calcium, respectively, and from lactate, which was 7.9 ± 0.2 and 5.5 ± 0.4 nmol per mg of cells (wet weight) in the presence or absence of calcium, respectively. •, $P < 0.001$ vs. basal value; ••, $P < 0.005$ vs. basal value.

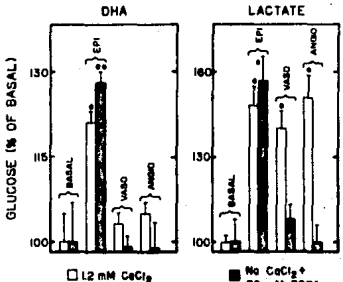

FIG. 3. Effect of 1 μM epinephrine and 1 μM propranolol (EPI), 1 milliunit of vasopressin (VASO), and 1 μM angiotensin II (ANGIO) on the stimulation of gluconeogenesis from 2.5 mM dihydroxyacetone (DHA) and 10 mM lactate in medium containing 1.2 mM $CaCl_2$ (open bars) or 25 μM EGTA without $CaCl_2$ (hatched bars). Incubation conditions were the same as those described in Fig. 2. Results are the means (±SEM) of duplicate incubations from 4–8 cell preparations and are expressed as percentage of basal glucose synthesis. Basal values are given in the legend to Fig. 2. •, $P < 0.001$ vs. basal values; ••, $P < 0.005$ vs. basal value.

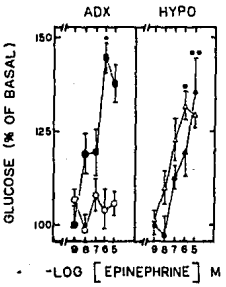

FIG. 4. Dose–response curves for the effect of epinephrine and 1 μM propranolol on gluconeogenesis from 2.5 mM dihydroxyacetone (open symbols) and lactate 10 mM (closed symbols). Hepatocytes isolated from adrenalectomized (ADX) or hypothyroid (HYPO) rats were incubated with substrates and agents for 60 min in the presence of 1.2 mM $CaCl_2$. Results are the means (±SEM) of duplicate incubations from 4–6 cell preparations and are expressed as the percentage of basal glucose synthesis, which was in cells from adrenalectomized rats, 16.2 ± 0.6 and 14.7 ± 0.9 from dihydroxyacetone and lactate, respectively, and in cells from hypothyroid rats, 14.9 ± 0.87 and 9.2 ± 0.85 nmol per mg of cell wet weight from dihydroxyacetone and lactate, respectively. •, $P < 0.001$ vs. basal value; ••, $P < 0.02$ vs. basal value.

agonist in liver cells (24, 25), stimulated in a dose-dependent fashion gluconeogenesis from lactate, but it was completely ineffective in doing so when dihydroxyacetone was the substrate (Fig. 6).

DISCUSSION

Several lines of evidence have suggested that some differences in the action of vasopressin and angiotensin II may

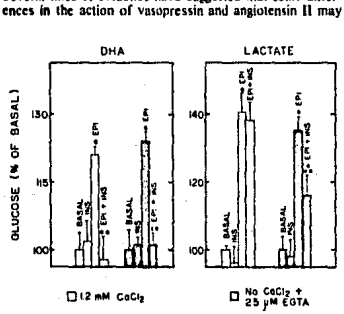

FIG. 5. Effect of insulin on stimulation of gluconeogenesis from 2.5 mM dihydroxyacetone (DHA) and 10 mM lactate. Hepatocytes from control rats were incubated in medium containing 1.2 mM $CaCl_2$ (open bars) or 2.5 μM EGTA without $CaCl_2$ (hatched bars) in the presence of 1 μM epinephrine and 1 μM propranolol (EPI), 1 μM epinephrine/1 μM propranolol/0.1 μM insulin (EPI + INS) or 0.1 μM insulin (INS). •, $P < 0.001$ vs. basal value; ••, $P < 0.001$ vs. EPI; •••, $P < 0.02$ vs. EPI.

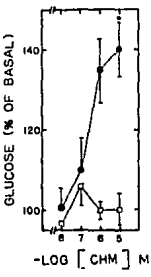

FIG. 6. Dose–response curve for effect of cycloheximide (CHM) on gluconeogenesis from 2.5 mM dihydroxyacetone (open squares) or 10 mM Lactate (closed circles). •, $P < 0.001$ vs. basal value.

exist, and we have proposed for the hypothesis that α_1-adrenergic action involves two mechanisms—i.e., the "conventional" mechanism shared with vasopressin and angiotensin II and an "alternative" mechanism (see Fig. 1) (9–14). Gluconeogenesis from lactate seems to be modulated by both mechanisms. In contrast, synthesis of glucose from dihydroxyacetone seems to be exclusively modulated by one of the pathways of the α_1-adrenergic action—i.e., the alternative pathway. Several criteria were fulfilled for this conclusion: (*i*) the effect of epinephrine is not mimicked by vasopressin, angiotensin II, or cycloheximide (Figs. 3 and 6); (*ii*) this action of epinephrine is not dependent on the presence of extracellular calcium (in fact, the effect is even bigger in the absence of extracellular calcium; Fig. 2); (*iii*) it is very sensitive to the action of insulin (Fig. 5); and (*iv*) it can be observed in hepatocytes from hypothyroid rats but not in cells from adrenalectomized animals (Fig. 4).

Our results are in close agreement with those of Kneer and Lardy (15). However, there is a difference in our findings; these authors did not observe an effect of adrenergic agents on gluconeogenesis from lactate in the absence of calcium (15). The reason for this is unclear at present. These authors used norepinephrine rather than epinephrine, and only at one concentration.

The effect of epinephrine on gluconeogenesis from lactate seems to be mediated by both pathways of α_1-adrenergic action but, interestingly, the dose–response curve to the agonist is shifted to the left in the absence of calcium as compared to the control (Fig. 2). This is surprising because actually we expected the opposite to occur, and it suggests that some amplification of the α_1-adrenergic action may take place under this condition. The effects of α_1-adrenergic agents are thought to occur through mechanisms independent of cyclic AMP (1–8). However, when the cells are incubated in the absence of calcium, the situation is somewhat more complicated. Under this condition, α_1-adrenergic activation reportedly increases cyclic AMP levels (23, 28, 29). Furthermore, it has been suggested that α_1-adrenoceptors become simultaneously coupled to two signal transduction mechanisms: calcium mobilization and cyclic AMP generation (19, 30). Therefore, a role of cyclic AMP in α_1-adrenergic action cannot be ruled out at the present. However, we have been unable to detect any significant stimulation of cyclic AMP formation by α_1-adrenergic activation, even in cells from hypothyroid rats where the alternative α_1-adrenergic pathway predominates (12). In addition, Lardy *et al.* (31) have observed that atractyloside inhibits the enhancement of

gluconeogenesis from dihydroxyacetone produced by cyclic AMP or glucagon but not the enhancement produced by epinephrine. These data raise some doubts on the metabolic significance of the reported α_1-adrenergic stimulations of cyclic AMP generation.

In summary, the data are consistent with our proposal of two mechanisms or pathways involved in α_1-adrenergic action. They also suggest that the α_1-adrenergic regulation of gluconeogenesis from dihydroxyacetone takes place through the pathway that is calcium-independent, insulin-sensitive, and modulated by glucocorticoids.

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"α_1-ADRENERGIC DESENSITIZATION INDUCED BY PHORBOL ESTERS, VASOPRESSIN AND ANGIOTENSIN II IN RAT HEPATOCYTES"

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SUMMARY

Using isolated rat hepatocytes the effect of vasopressin, angiotensin II and phorbol myristate acetate on the action of epinephrine + propranolol ($alpha_1$-adrenergic action), was studied. Three conditions in which epinephrine + propranolol produces clear metabolic effects but the vasopressor peptides do not do it so (although they stimulate phosphoinositid turnover) were selected. These conditions are: a) ureagenesis in medium without added calcium and containing 25 μM EGTA, b) ureagenesis using cells from hypothyroid animals and c) gluconeogenesis from dihydroxyacetone. It was observed that under the three conditions these agents inhibited in a concentration-dependent fashion the effect of epinephrine + propranolol. It is suggested that activation of protein kinase C by phorbol esters or physiological stimuli (hormones that activate phosphoinositide turnover, such as vasopressin or angiotensin II) modulate the hepatocyte $alpha_1$-adrenergic responsiveness.

1.- INTRODUCTION

Activation of α_1-adrenoceptors results in an increased turnover of phosphoinositides with the generation of diacylglycerols and inositol 1, 4, 5 trisphosphate; the latter metabolite induces mobilization of calcium [1-3]. Calcium, inositol trisphosphate and diacylglycerols are putative second messengers or coupling factors of the α_1-adrenergic action [1-4]. Propagation of the signal seems to occur through calcium-dependent protein kinases and the calcium-phospholipid-dependent kinase (protein kinase C)[5]. Surprisingly, activation of protein kinase C with phorbol esters not only does not mimic the metabolic effects of epinephrine but abolishes the action of α_1-adrenergic agonists in hepatocytes [6-10]. This raises the question as to whether physiological stimuli may produce a similar effect. Here we describe experiments designed to test this point. Our results clearly indicate that the action of hormones that activate phosphoinositide turnover, such as vasopressin and angiotensin II, leads to α_1-adrenergic desensitization.

2.- MATERIALS AND METHODS

The sources of materials are those reported previously [6-8]. Hepatocytes were isolated by the method of Berry and Friend [11] from female Wistar rats weighing 180-200 g. Animals fed *ad libitum* were used for the studies on ureagenesis and 24-hours fasted rats in those on gluconeogenesis. Cells (roughly 40 mg wet weight) were incubated in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, under an atmosphere of 95% O_2 5% CO_2, for 60 min at 37°C in a water bath shaker. For the studies

of gluconeogenesis the cells were incubated in the presence of 2.5 mM dihydroxyacetone (DHA) and glucose was determined in aliquots of the supernatant by the glucose oxidase-peroxidase method [12]. Glucose production from endogenous substrates was substracted from the values obtained in the presence of DHA. In the studies of ureagenesis, the medium in which the cells were incubated was supplemented with 2 mM ornithine and 10 mM glutamine. Urea was quantified in the supernatant by the method of Gutman and Bergmeyer [13]. Buffer without calcium refers to Krebs-Ringer bicarbonate buffer to which no calcium chloride was added and was supplemented with 25 μM EGTA.

In all experiments in which epinephrine was employed, the cells were also incubated in the presence of 10^{-6} or 10^{-5} M propranolol to block its β-adrenergic activity. Propranolol by itself did not affect the parameters studied. Hypothyroidism was induced by giving the animals water containing 0.030% 6-n-propyl 2-thiouracil for 30-50 days and it was assessed by decreased weight gain, dryness of the fur and decreased levels of triiodothyronine [14,15].

3.- RESULTS AND DISCUSSION

In hepatocytes, epinephrine (α_1-adrenergic effect), vasopressin and angiotensin II stimulate phosphoinositide turnover and some metabolic pathways (glycogenolysis [16], gluconeogenesis [17-19] and ureagenesis [20]). However there are at least three conditions in which, although phosphoinositide turnover is stimulated by vasopressin and angiotensin II, no metabolic stimulation of the metabolic pathways is produced by the vasopressor peptides. These conditions are: a) incubations in the absence of calcium [20]; b) cells from hypothyroid animals [14,15] and

c) gluconeogenesis from DHA [18,19]. Interestingly, in this three conditions α_1-adrenergic agonists clearly stimulate the metabolic parameters, situation which has allowed us to propose the involvement of two parameters, one shared and another not shared with the vasopressor peptides, in the α_1-adrenergic action [14,15,19-22]. Activation of protein kinase C by phorbol diesters blocks both pathways of the α_1-adrenergic action [6,7,19,22]. Taking advantage of this situation, we examined the effect of 4-β-phorbol 12-myristate 13-acetate (PMA), vasopressin and angiotensin II on the action of epinephrine plus propranolol.

Epinephrine, (in the presence of 10^{-6}M propranolol) stimulated in a concentration dependent fashion the production of glucose from DHA (Fig.1, panel A). In agreement with previous findings PMA, vasopressin and angiotensin II were unable to alter the basal rate of glucose production from DHA (not shown)[18,19]. Interestingly, these three agents inhibited in a concentration-dependent fashion the effect of 10^{-6}M epinephrine (plus 10^{-6}M propranolol). The order of potency for this effect was vasopressin > PMA = angiotensin II (Fig. 1, panel B).

Similar results were obtained when ureagenesis was studied. Epinephrine (in the presence of 10^{-5}M propranolol) stimulated ureagenesis in cells from control rats incubated in the absence of calcium (Fig. 2, panel A), and in cells from hypothyroid rats (Fig. 3, panel A). Basal production of urea was not affected by vasopressin, angiotensin II or PMA under these conditions (not shown)[14,15,20] but clearly inhibited in a concentration-dependent fashion the effect of epinephrine (plus propranolol) (Fig. 2, panel B and Fig. 3, panel B). The order of potency for these effects was also vasopressin > PMA > angiotensin II (Fig. 2, panel B and Fig. 3, panel B).

Our data suggest that the activity of protein kinase C modulates the α_1-adrenergic sensitivity of hepatocytes. The physiological activators of protein kinase C are thought to be diacylglycerols which are formed from the breakdown of phosphatidylinositol [4]. Vasopressin transiently increases the concentration of diacylglycerols in hepatocytes [23] and it has been observed that a synthetic diacylglycerols (1-oleoyl 2-acetyl glycerol) can block the α_1-adrenergic action in liver cells [10].

Activation of protein kinase C seems to play a key role in modulating the cell sensitivity to a variety of hormones, neurotransmitters and growth factors including insulin [24], epidermal growth factor [25,26] acetyl choline (muscarinic [27,28] and adrenaline [6-10, 28-31]. Most of these actions of protein kinase C lead to cell desensitization. In this sense protein kinase C seems to play a key role in homologous and heterologous desensitization. However, this is not the case for all modulations of cell responsiveness. In a very elegant study, Sugden et al. [31], observed that α_1-adrenergic activation potentiates β-adrenergic action in rat pinealocytes [31]. This effect is mimicked by PMA [31]. Most of these phorbol ester-mediated changes in cell responsiveness to hormones seem to be associated to receptor phosphorylation [24-30]. The underlying molecular mechanism(s) through which phorbol esters, vasopressin and angiotensin II block α_1-adrenergic action is currently under research.

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Fig. 1 Concentration-response Curve for the Effect of Epinephrine on Gluconeogenesis from 2.5 mM Dihydroxyacetone (panel A) and Effects of TPA, Vasopressin and Angiotensin II (panel B). Hepatocytes from fasted 24 hours animals were incubated in the presence of different concentrations of epinephrine (plus 10^{-6} M propranolol (o---o) (panel A), or with 10^{-6} M epinephrine + 10^{-6} M propranolol and different concentrations of TPA (●-●), vasopressin (▫-▫) or angiotensin II (▪-▪) (panel B). Results are expressed as percentage of basal glucose synthesis which was 13.5 ± 2 nmol/mg cell wet weight. Plotted are the means and vertical lines represents the S.E.M. of duplicate incubations from 3-6 cell preparations.

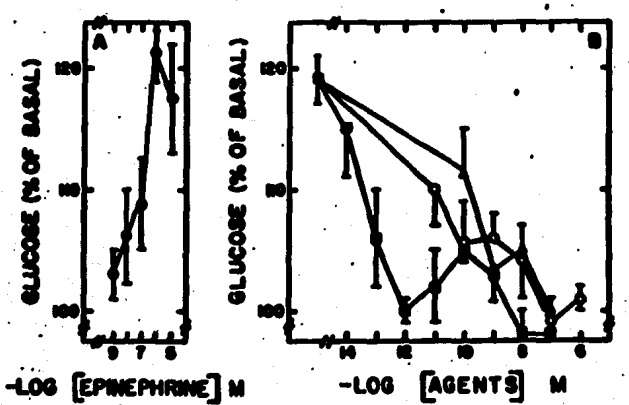


Fig. 2. Concentration-response Curve for the Effect of Epinephrine on Ureagenesis (panel A) and Effects of TPA, Vasopressin and Angiotensin II (panel B). Hepatocytes were incubated for 60 min in medium without $CaCl_2$ and containing 25 μM EGTA in the presence of different concentrations of epinephrine (plus 10^{-6} M propranolol) (e-e), (panel A) or with 10^{-5} M epinephrine + 10^{-6} M propranolol and different concentrations of TPA (▲), vasopressin (▽) and angiotensin II (o-o) (panel B). Results are the means (± S.E.M.) of duplicate incubations from 3-8 cell preparations and are expressed as percentage of basal urea synthesis, which was 27 ± 4 nmol/mg cell wet weight.

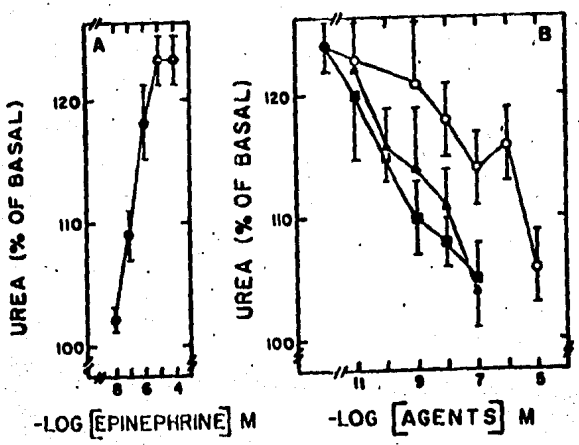
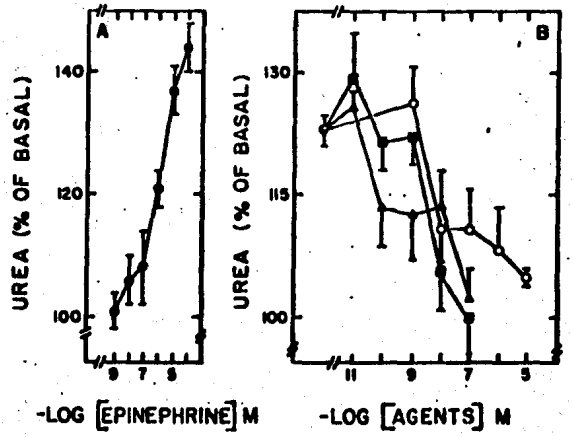


Fig. 3 Concentration-response Curve for the Effect of Epinephrine on Ureagenesis (panel A) and Effects of TPA, Vasopressin and Angiotensin II (panel B) in Hepatocytes isolated from Hypothyroid Rats. Hepatocytes were incubated for 60 min in the presence of different concentrations of Epinephrine (plus 10^{-6} M propranolol) (e-e) (panel A) and with 10^{-6} M epinephrine + 10^{-6} M propranolol and different concentrations of TPA (Δ), vasopressin (☐) or angiotensin II (o-o) (panel B). Results are the means (S.E.M.) of duplicate incubations from 3-5 cell preparations and are expressed as the percentage of basal urea synthesis, which was 14.5 ± 1 nmol/mg cell wet weight.



HOMOLOGOUS AND HETEROLOGOUS DESENSITIZATION OF ONE OF THE PATHWAYS OF THE ALPHA$_1$-ADRENERGIC ACTION. EFFECTS OF EPINEPHRINE, VASOPRESSIN, ANGIOTENSIN II AND PHORBOL 12-MYRISTATE 13-ACETATE.

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Key words:

- Alpha$_1$-adrenergic
- Protein kinase c
- Desensitization

SUMMARY

Activation of protein kinase c blocks the alpha$_1$-adrenergic action in hepatocytes. Preincubation of hepatocytes (in buffer with or without calcium) with vasopressin, angiotensin II, phorbol myristate acetate (PMA) or epinephrine + propranolol markedly diminished the alpha$_1$-adrenergic responsiveness of the cells (stimulation of ureagenesis) assayed in buffer without calcium. On the contrary, when the alpha$_1$-adrenergic responsiveness was assayed in buffer containing calcium no effect of the preincubation with vasopressin, angiotensin II or PMA was observed; preincubation with epinephrine diminished the alpha$_1$-adrenergic responsiveness of the cells.

In hepatocytes from hypothyroid rats the preincubation with the activators of protein kinase c (vasopressin, angiotensin II, PMA and epinephrine) reduced markedly the alpha$_1$-adrenergic responsiveness of the cells whereas in identical experiments using cells from adrenalectomized rats only the preincubation with epinephrine diminished the responsiveness.

It is concluded that activation of protein kinase c induces desensitization of the alpha$_1$-adrenergic action in hepatocytes and that the calcium-independent pathway of the alpha$_1$-adrenergic action (predominant in cells from hypothyroid animals) resensitizes more slowly than the calcium-dependent pathway (predominant in cells from adrenalectomized rats). Epinephrine in addition to induce this type of desensitization (through protein kinase c) induces a further refractoriness of the cells towards alpha$_1$-adrenergic agonists.

INTRODUCTION

One of the most fascinating aspects of biology is the dynamic nature of cell responsiveness: cells have acquired a large number of adaptative processes to modulate their responses to hormones, neurotransmitters, autacoids and in general, to external stimuli. One of such adaptative processes is the phenomenon of desensitization, in which a cell becomes refractory towards a hormonal agonist after a single or repetitive stimulation. Desensitization may be hormone specific (homologous) when exposure of cells to a given hormone results in subsequent decreased response to the same hormone or may be non-specific (heterologous) when the response to other hormones is diminished. The process may occur rapidly (minutes or seconds) or more slowly (hours to days) and may involve changes at the receptor level or distal to the receptor.

Desensitization has been mainly reported for hormones that act through receptors coupled to adendylate cyclase such as the beta-adrenergic receptor [reviewed in 1 and 2]. However, there is also some evidence for desensitization of receptors not coupled to adenylate cyclase [3-9]. In liver cells, it has been reported that vasopressin and alpha$_1$-adrenergic agonists induce dose-dependent heterologous desensitization [6-8]. However, other reports [9,10] indicated that no desensitization occurs in the presence of extracellular calcium, suggesting that the desensitization observed [6-8] could reflect the depletion of a hormone-sensitive calcium pool [10].

We [11-15] and others [16-17] have observed that activation of protein kinase c by phorbol esters or synthetic diacyl glycerols blocks the action of alpha$_1$-adrenergic agents but not that of other agents that act through the same process of signal transduction (such

as vasopressin and angiotensin II). We have suggested [11-15], that activation of protein kinase c by phorbol esters may lead to refractoriness through alpha$_1$-adrenergic receptor phosphorylation; direct evidence supporting this suggestion has been recently reported [18].

Protein kinase c activation seems to be part of the signal propagation process for the action of agents that stimulates phosphoinositide turnover [19-22]. Theoretically therefore, activation of phosphoinositide turnover by alpha$_1$-adrenergic agents, vasopressin or angiotensin II could lead to protein kinase c activation and blockade of the alpha$_1$-adrenergic action. We have recently tested this point studying conditions in which the alpha$_1$-adrenergic action is preserved but there is no metabolic effect of the vasopressor peptides vasopressin and angiotensin II (although they stimulates phosphoinositide turnover) [23]. Under these conditions phorbol esters, vasopressin and angiotensin II produce a dose-dependent inhibition of the alpha$_1$-adrenergic action [27].

The effect of removal of these ligands (phorbol esters, epinephrine, vasopressin and angiotensin II) on the alpha$_1$-adrenergic responsiveness of the hepatocytes was studied and the results are here presented. We have previously suggested that the alpha$_1$-adrenergic action in liver cells involves two pathways: a) a pathway shared with vasopressin and angiotensin II and which is calcium-dependent, insulin-insensitive and modulated by thyroid hormones (i.e. markedly diminished in hypothyroid rats) and b) another pathway not shared with the vasopressor peptides, independent of extracellular calcium, insulin-sensitive and modulated by glucocorticoids (markedly

diminished in adrenalectomized rats) [13, 24-28]. Our data indicate that after removal of the activators of protein kinase c the calcium dependent pathway recovers rapidly whereas the calcium-independent pathway does not dc it so.

MATERIALS AND METHODS

l-Epinephrine, dl-propranolol, urease, arginine-vasopressin, angiotensin II, 6-n-propyl 2-thiouracil, EGTA and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Company. Collagenase was from Worthington and [^{32}P]Pi (carrier free) was obtained from New England Nuclear. Glucagon was a generous gift from Eli Lilli. Other substances were reagent grade of the best quality available.

Female Wistar rats (180-200 g) fed ad libitum were used. Hypothyroidism was induced by giving the rats water containing 0.03% 6-n-propyl 2-thiouracil for 40-50 days [24,25]. Hypothyroidism was assessed by decreased weight gain, dryness of the fur and low levels of triiodothyronine [25]. Bilateral adrenalectomy was performed by a dorsal approach; adrenalectomized animals were given 0.85% NaCl to drink and were used 5-8 days after surgery [26].

Hepatocytes were isolated by the method of Berry and Friend [29]. Hepatocytes were incubated in Krebs-Ringer bicarbonate buffer under an atmosphere of 95% O_2 5% CO_2, pH 7.4. Cells were incubated without any agent or with maximally effective concentrations of PMA (10^{-7}M), vasopressin (10^{-7}M), angiotensin II (10^{-8}M) or epinephrine (10^{-4}M + 10^{-6}M propranolol) for 15 minutes to induce desensitization. After this preincubation, the cells were centrifuged and washed twice with Krebs-Ringer bicarbonate buffer.

Cells were resuspended and the effect of hormones tested (60 minutes of incubation). In some experiments buffer without calcium was used which refers to Krebs-Ringer bicarbonate buffer to which no calcium chloride was added and was supplemented with 25 µM EGTA. In all the experiments in which epinephrine was used 10^{-6}M propranolol

was included to block its beta-adrenergic activity. Propranolol by itself did not affected the parameters studied. For the study of ureagenesis the medium was supplemented with 10 mM glutamine and 2 mM ornithine. Urea was quantified in cell supernatants by the method of Gutman and Bergmeyer [30]. For the study of phosphatidylinositol labeling, the cells were incubated in the presence of 10 μ Ci/ml [^{32}P]Pi for 60 minutes. Cell lipids were extracted with chloroform/methanol (2:1) and phospholipids separated by one-dimension thin-layer chromatography [31]. Radioactivity was counted in silica gel scrapings of each phospholipid.

RESULTS

A) Desensitization and incubation in the absence of calcium: In the experiments presented in Fig. 1, liver cells were preincubated for 15 min with the indicated agents (none, PMA, vasopressin, angiotensin II or epinephrine + propranolol) in buffer without calcium. The cells were then washed and incubated also in buffer without calcium. It can be observed that the cells that were preincubated without any agent responded in a dose-dependent fashion to epinephrine + propranolol increasing the synthesis of urea (Fig. 1, panel A). However, cells preincubated with PMA, vasopressin, angiotensin II or epinephrine + propranolol became refractory to the action of epinephrine + propranolol (Fig. 1, panel A). Such refractoriness to the alpha$_1$-adrenergic stimulation was not due to a general damage of the cells since these same cells clearly responded to 10^{-7}M glucagon (Fig. 1, panel B).

B) Desensitization in the absence of calcium and incubation in the presence of calcium. In these experiments the cells were preincubated as indicated above but were washed and incubated in buffer containing calcium chloride. Under these conditions, the concentration-response curves for epinephrine of cells preincubated in the absence of agents, PMA, vasopressin and angiotensin II were nearly identical (Fig. 2, panel A). However, the concentration-response curve to epinephrine of cells preincubated with the adrenergic agonist was shifted to the right and rather flat (Fig. 2, panel A): the response to glucagon of these cells was also somehow diminished (Fig. 2, panel B).

C) Desensitization in the presence of calcium and incubation in the

absence of calcium. Cells were preincubated in buffer containing calcium in order to define if a depletion of calcium during the preincubation could be responsible of the blunted response to alpha$_1$-adrenergic stimulation. After the preincubation the cells were washed and incubated in buffer without calcium and containing 25 μM EGTA. Under these conditions, the response to epinephrine + propranolol was markedly reduced in cells preincubated with PMA, vasopressin, angiotensin II or epinephrine as compared to the cells preincubated without any of these agents (Fig. 3, panel A). The effect of glucagon on these cells is presented in Fig. 3, panel B.

Some experiments were performed in the following way: cells were pre-incubated in buffer without calcium with or without the desensitizing agent (vasopressin), washed and incubated in buffer containing calcium for 15 min to allow re-uptake of any calcium lost and then washed and incubated in buffer without calcium. Under these conditions pretreatment with vasopressin, markedly diminished the effect of alpha$_1$-adrenergic stimulation (data not shown).

D) Phosphatidylinositol turnover. It is generally accepted that phosphoinositide turnover is involved in the mechanism of action of alpha$_1$-adrenergic amines [22, 32, 33]. We studied the labeling of phosphatidylinositol with [^{32}P]Pi as an index of phosphoinositide turnover. It was observed that preincubation and incubation in the absence (Fig. 4) or presence (Fig. 5) of calcium resulted in identical responses i.e. preincubation of the cells with PMA, vasopressin or angiotensin II did not affect the stimulation of phosphatidylinositol labeling induced by epinephrine; however, cells pre-incubated with

epinephrine presented a diminished response to alpha$_1$-adrenergic activation as compared to the control cells (Figs. 4 and 5). The effect of vasopressin on phosphatidylinositol labeling is presented for comparison (Figs. 4 and 5, panels B).

E) Studies with cells from adrenalectomized rats and from hypothyroid rats. In these studies the cells were preincubated, washed and incubated in buffer containing calcium. It was observed that in cells from adrenalectomized rats, preincubation with PMA, vasopressin and angiotensin II did not modify substantially the concentration-response curve to epinephrine + propranolol as compared to cell preincubated without any of these agents (Fig. 6, panel A). Only the response to epinephrine was significantly decreased in cells preincubated with this adrenergic amine (Fig. 6, panel A).

On the contrary, in cells from hypothyroid rats (Fig. 7) preincubation with PMA, vasopressin or angiotensin II clearly diminished and with epinephrine nearly abolished the effect of alpha$_1$-adrenergic stimulation (Fig. 7). The effect of 10^{-7}M glucagon is presented for comparison (Fig. 7, panel B).

DISCUSSION

The finding that activation of protein kinase c by phorbol esters blocks the alpha$_1$-adrenergic responsiveness in liver cells [11-18] has several implications. One of the most obvious is its physiological significance [see the comment in 34]: protein kinase c seems to be involved in the action of agents that act through phosphoinositide turnover and calcium signalling [19-22] such as alpha$_1$-adrenergic agents, vasopressin and angiotensin II. In our previous publication [23] we reported that vasopressin and angiotensin II block in a concentration-dependent fashion the alpha$_1$-adrenergic action (calcium-independent pathway). These data clearly indicate that physiological stimuli can induce the refractoriness (or desensitization) towards alpha$_1$-adrenergic agonists. However, these studies were performed with tonic activation of protein kinase c (i.e. continuous presence of the agonists).

Removal of these activators of protein kinase c evidenced two types of responses. In cells (from normal animals) incubated in the presence of calcium or in cells from adrenalectomized rats the responsiveness to alpha$_1$-adrenergic agonists was recovered. On the contrary, in cells (from normal rats) incubated in the absence of calcium or in cells from hypothyroid rats the alpha$_1$-adrenergic responsiveness was not recovered during the incubation.

Activation of protein kinase c by phorbol esters in cells (from control animals) incubated in the presence of calcium or in cells from adrenalectomized animals blocks the alpha$_1$-adrenergic actions [11-18]. We have no evidence that under these conditions, vasopressin or angiotensin II would also block the alpha$_1$-adrenergic action (the point is untestable measuring phosphoinositide turnover or metabolic

parameters since the vasopressor peptides have effects by themselves); however, it seems reasonable to assume that it occurs so. The data using cells (from normal animals) incubated with calcium and with cells from adrenalectomized rats indicate that the process of desensitization is readily reversible and suggest that the phosphorylated alpha$_1$-adrenergic receptor [18] is rapidly resensitized possibly through the action of one of several very active phosphatases. The underlying cellular mechanism of the action of the activators of protein kinase c on the alpha$_1$-adrenoceptor is far from clear. We [15] and others [16] have been unable to detect any change in the affinity for agonists or antagonists of alpha$_1$-adrenoceptors in membranes obtained from livers treated with phorbol esters as compared to the controls. Lynch et al [16] observed that phorbol esters induce a 30-40% decrease in the number of alpha$_1$-adrenoceptors and we [15] observed a decrease in affinity for agonists in whole cells (but not in membranes). It is possible that the reactivation process could have made the changes less apparent. It is also possible that several cellular processes may contribute to produce the effect (receptor phosphorylation, change in affinity, internalization and recycling, etc.). Evidence for this has been obtained in studies on the transferring receptor [35]: it has been observed that phorbol esters induce receptor phosphorylation and internalization and that removal of the phorbol ester lead the receptor to cycle back to the plasma membrane [35]. How general this observation is and its relationship with what we observe for the alpha$_1$-adrenergic action remains to be determined.

Cells from normal rats incubated in the absence of calcium and

cells from hypothyroid rats remained desensitized towards alpha$_1$-adrenergic agonists for longer time. The reason for this difference is unclear. We have suggested that in hepatocytes the alpha$_1$-adrenergic action involves two pathways: a) one of them calcium-dependent and modulated by thyroid status (i.e. absent in cells from hypothyroid animals but predominant in cells from adrenalectomized rats); this pathway seems to resensitize rapidly and b) another pathway calcium-independent and modulated by glucocorticoids (i.e. absent in cells from adrenalectomized but predominant in cells from hypothyroid rats); this pathway seems to recover more slowly. The present data are consistent, although do not prove, our hypothesis. The possibility that such desensitization could be secondary to depletion of calcium was considered. However several facts argue against this possibility: a) desensitization of this calcium-independent pathway in cells from normal animals was observed even when the preincubation was performed in medium containing calcium; b) when the cells were preincubated with the desensitizing agent (vasopressin) in the absence of calcium a second pre-incubation with calcium (and without vasopressin) did not restore the calcium-independent pathway; c) the experiments with cells from hypothyroid animals were performed in buffer containing calcium at all times; d) phorbol esters do not seem to induce calcium mobilization [16,17].

It is clear from our experiments that preincubation with epinephrine induces a desensitization that is present under all conditions. These data suggest that alpha$_1$-adrenergic agonists may, in addition to induce refractoriness through protein kinase c activation, induce further desensitization secondary to receptor occupancy and activation. Studies are in progress to elucidate the mechanism/s)

involved.

It is clear that much research is required to reach a better understanding of the liver alpha$_1$-adrenergic phenomenon. We hope the preent data may contribute to it and stimulate further study.

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Fig. 1. CONCENTRATION-RESPONSE CURVES FOR THE EFFECT OF EPINEPHRINE ON UREAGENESIS IN HEPATOCYTES INCUBATED (IN THE ABSENCE OF CALCIUM) (PANEL A) AND EFFECTS OF GLUCAGON (PANEL B). Hepatocytes were preincubated for 15 min in the absence of calcium (plus 25 μM EGTA) with 10^{-7}M vasopressin (VASO, ■), 10^{-8}M angiotensin II (ANGIO, ●), 10^{-7}M PMA (▲), 10^{-6}M epinephrine plus 10^{-6}M propranolol (EPI, □) or buffer alone (CONTROL, O). After 15 min the cells were washed twice to remove the agents, and were incubated for 60 min (all in the absence of calcium plus 25 μM EGTA) in the presence of different concentrations of epinephrine + 10^{-6}M propranolol (panel A) or without agent (open bar), 10^{-6}M epinephrine + 10^{-6} propranolol (dotted bar) or 10^{-7}M glucagon (dashed bar) (panel B). Results are expressed as percentage of basal urea synthesis which is given above the control bars (panel B open bars) as nmols/mg cells wet weight. Plotted are the means and vertical lines represents the S.E.M. of duplicate incubations from 4-8 cell preparations.

Fig. 2. CONCENTRATION-RESPONSE CURVES FOR THE EFFECT OF EPINEPHRINE ON UREAGENESIS IN CONTROL AND DESENSITIZED HEPATOCYTES (PANEL A) AND EFFECTS OF GLUCAGON (PANEL B). Hepatocytes were preincubated for 15 min in absence of calcium plus 25 μM EGTA without (O) or with the desensitizing agents [vasopressin (■), angiotensin (●), PMA (▲) or epinephrine (□)]. After 15 min, the cells were washed twice to remove these agents, and were incubated for 60 min in buffer containing chloride calcium and with different concentrations of epinephrine + 10^{-6}M propranolol (panel A) or without any agent (open bar) epinephrine (dotted bar) or glucagon (dashed bar) (panel B). Results are expressed as percentage of basal urea synthesis. Plotted are the

means and vertical lines represent the S.E.M. of duplicate incubations from 4-8 cell preparations. Other indications as in Fig. 1

Fig. 3. CONCENTRATION-RESPONSE CURVE FOR THE EFFECT OF EPINEPHRINE (IN THE ABSENCE OF CALCIUM) ON UREAGENESIS IN HEPATOCYTES PREINCUBATED IN BUFFER CONTAINING CALCIUM. Hepatocytes were preincubated for 15 min in buffer containing calcium without (○) or with the desensitizing agents [vasopressin (■), angiotensin II (●), PMA (▲) or epinephrine (□)] and then washed and incubated (in buffer without calcium) with different concentrations of epinephrine + 10^{-5}M propranolol (panel A) or without agents (open bars), 10^{-5}M epinephrine + 10^{-5}M propranolol (dotted bars) or 10^{-7}M glucagon (dashed bars) (panel B). Other indications as in Fig. 1.

Fig. 4. CONCENTRATIONS-RESPONSE CURVES FOR THE EFFECT OF EPINEPHRINE (PANEL A) OR 10^{-7}M VASOPRESSIN (PANEL B) ON THE LABELING OF PHOSPHATIDYLINOSITOL (PI). Hepatocytes were preincubated for 15 min in absence of calcium plus 25 μM EGTA without (○) or with the desensitizing agents [vasopressin (■), angiotensin II (●), PMA (▲) or epinephrine (□)]. After 15 min the cells were washed twice and then incubated 60 min in the absence of calcium plus 25 μM EGTA in the presence of different concentrations of epinephrine + 10^{-5}M propranolol (panel A) or 10^{-7}M vasopressin (panel B). Plotted are the means ± S.E.M. of duplicate incubations from three cell preparations. Results are expressed as percentage of the basal incorporation of [^{32}P]Pi to PI, which was 75 ± 9, 34 ± 4, 100 ± 2, 87 ± 7, 96 ± 6 cpm/mg cell wet wt. in cells preincubated without any agent (○), 10^{-7}

PMA (▲), 10^{-7}M vasopressin (■), 10^{-8}M angiotensin II (●), or 10^{-6}M epinephrine plus 10^{-5}M propranolol (□), respectively.

Fig. 5. DOSE-RESPONSE CURVES OF THE EFFECT OF EPINEPHRINE (PANEL A) OR 10^{-7}M VASOPRESSIN (PANEL B) ON THE LABELING OF PHOSPHATIDYLINOSITOL (PI). Hepatocytes were preincubated for 15 min in the absence of calcium plus 25 μM EGTA with the agents. After 15 min the cells were washed twice and are incubated 60 min (in buffer containing calcium chloride) in the presence of different concentrations of epinephrine + 10^{-5}M propranolol (panel A) or 10^{-7}M vasopressin (panel b). Plotted are the means ± S.E.M. of duplicate incubations from three cell preparations. Results are expressed as percentage of the basal incorporation of [^{32}P]Pi into PI, which was 117 ± 19, 123 ± 12; 125 ± 5, 133 ± 10, 150 + 30 cpm/mg cell wet wt. in cells preincubated without any agent (○), 10^{-7}M PMA (▲), 10^{-7}M vasopressin (■), 10^{-8}M angiotensin II (●), or 10^{-6}M epinephrine plus 10^{-5}M propranolol (□), respectively.

Fig. 6. CONCENTRATION-RESPONSE CURVE FOR THE EFFECT OF EPINEPHRINE (PLUS 10^{-5}M PROPRANOLOL) ON UREAGENESIS IN HEPATOCYTES OBTAINED FROM ADRENALECTOMIZED RATS. Hepatocytes obtained from adrenalectomized rats were preincubated for 15 min in buffer without (○) or with the desensitizing agents (vasopressin (■), angiotensin II (●), PMA (▲) or epinephrine (□)) and then washed and incubated with different concentrations of epinephrine + 10^{-5} propranolol (panel A) or without agents (open bars), 10^{-5}M epinephrine + 10^{-5}M propranolol (dotted bars) or 10^{-7}M glucagon (dashed bars) (panel B). Other indications as in Fig. 1.

Fig. 7. CONCENTRATION-RESPONSE CURVE FOR THE EFFECT OF EPINEPHRINE (PLUS 10^{-6}M PROPRANOLOL) ON UREAGENESIS IN HEPATOCYTES OBTAINED FROM HYPOTHYROID RATS. Hepatocytes obtained from hypothyroid rats were preincubated for 15 min in buffer without (○) or with the desensitizing agents [vasopressin (■), angiotensin II (●), PMA (▲) or epinephrine (□)] and then washed and incubated with different concentrations of epinephrine + 10^{-6} propranolol (panel A) or without agents (open bars), 10^{-4}M epinephrine + 10^{-6}M propranolol (dotted bars) or 10^{-7}M glucagon (dashed bars) (panel B). Other indications as in Fig. 1.

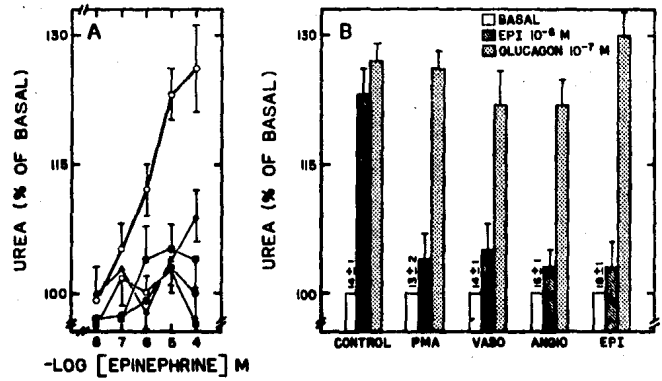
A
B
UREA (% OF BASAL)
130
115
100
8 7 6 5 4
-LOG [EPINEPHRINE] M
BASAL
EPI 10⁻⁶ M
GLUCAGON 10⁻⁷ M
CONTROL
PMA
VASO
ANGIO
EPI

FIG. 1

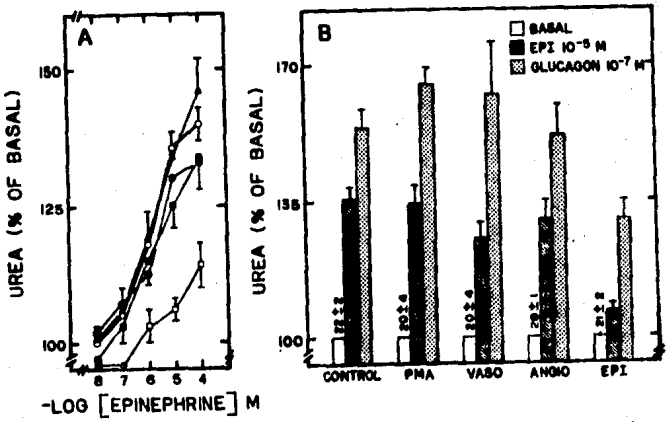
A
B
UREA (% OF BASAL)
150
125
100
8 7 6 5 4
-LOG [EPINEPHRINE] M
BASAL
EPI 10⁻⁵ M
GLUCAGON 10⁻⁷ M
170
135
CONTROL
PMA
VASO
ANGIO
EPI

FIG.2

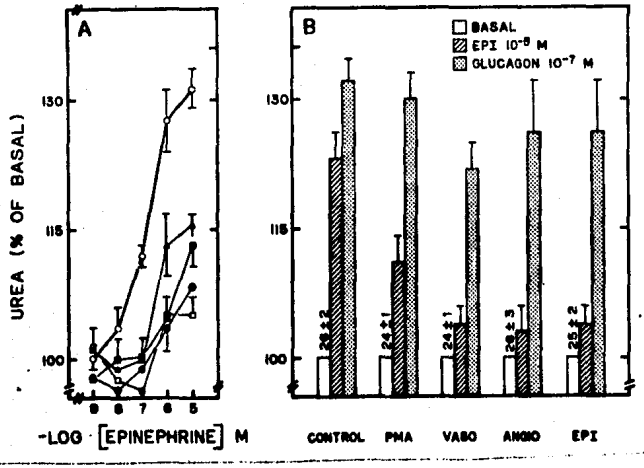
A
B
UREA (% OF BASAL)
130
115
100
BASAL
EPI 10⁻⁹ M
GLUCAGON 10⁻⁷ M
28±2
24±1
24±1
28±3
25±2
-LOG [EPINEPHRINE] M
CONTROL
PMA
VASO
ANGIO
EPI

FIG. 3

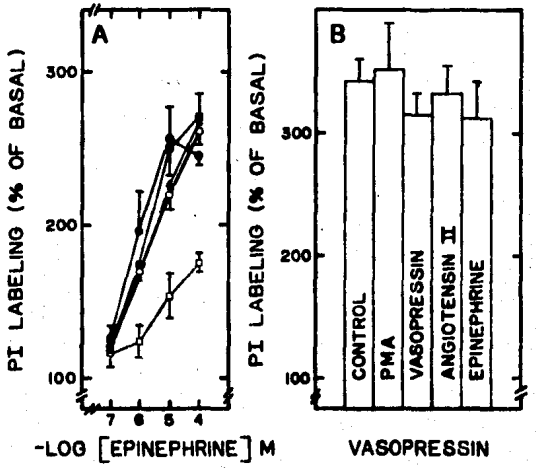
A
B
PI LABELING (% OF BASAL)
300
200
100
-LOG [EPINEPHRINE] M
CONTROL
PMA
VASOPRESSIN
ANGIOTENSIN II
EPINEPHRINE
VASOPRESSIN

FIG. 4

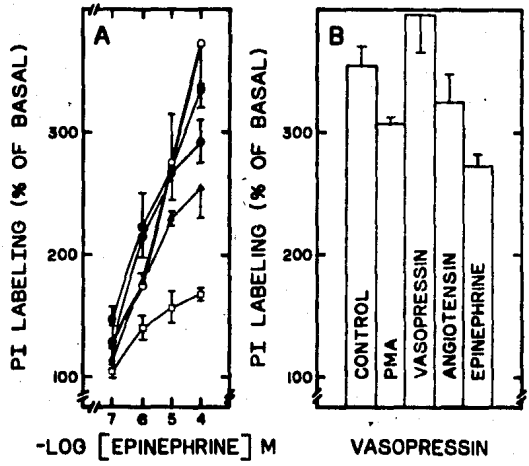
A
B
PI LABELING (% OF BASAL)
300
200
100
7
6
5
4
-LOG [EPINEPHRINE] M
CONTROL
PMA
VASOPRESSIN
ANGIOTENSIN
EPINEPHRINE
VASOPRESSIN

FIG. 5

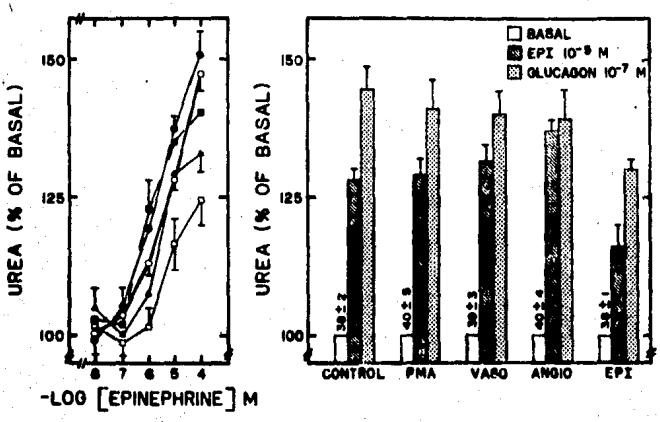
UREA (% OF BASAL)
150
125
100
6
7
6
5
4
-LOG [EPINEPHRINE] M
BASAL
EPI 10⁻⁹ M
GLUCAGON 10⁻⁷ M
38±2
40±5
38±3
40±4
38±1
CONTROL
PMA
VASO
ANGIO
EPI

FIG. 6

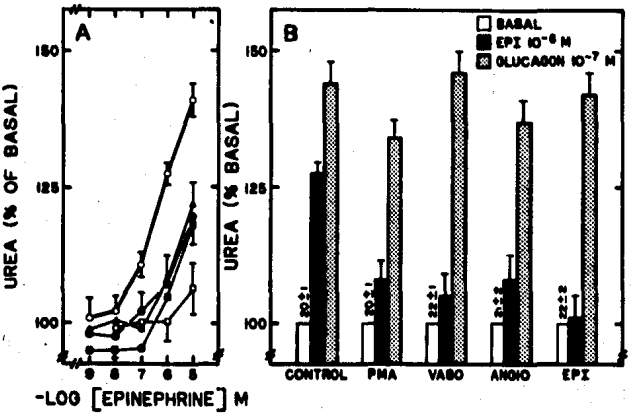
A
B
UREA (% OF BASAL)
UREA (% BASAL)
150
125
100
9
8
7
6
5
-LOG [EPINEPHRINE] M
BASAL
EPI 10⁻⁶ M
GLUCAGON 10⁻⁷ M
20±1
20±1
22±1
20±2
22±2
CONTROL
PMA
VASO
ANGIO
EPI

FIG. 7