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REGULACIÓN DE LA SECRECIÓN DEL FACTOR DE CRECIMIENTO NERVIOSO EN CÉLULAS GLIALES. MECANISMOS DE TRANSDUCCIÓN A TRAVÉS DE MENSAJEROS LIPÍDICOS

TESIS DOCTORAL

por

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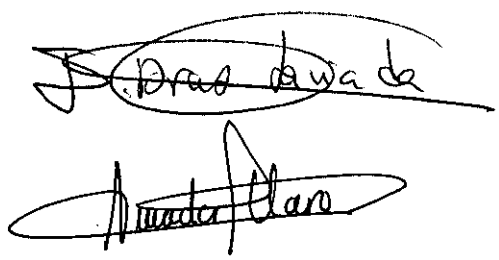

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2. Regulación de la proteína quinasa C ζ (PKC ζ) por mensajeros lipídicos en células gliales

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2.1 "Addition of phosphatidylcholine-phospholipase C induces cellular redistribution and phosphorylation of protein kinase C ζ in C6 glial cells"
Neurosci. Letters 219:68-70 (1996)

2.2 "Ceramide-induced translocation of protein kinase C ζ in primary cultures of astrocytes"
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3. Regulación de la síntesis y secreción de NGF por el factor de necrosis tumoral (TNFα) y lipopolisacárido (LPS) en astrocitos en cultivo primario

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J. Neurochem., en prensa

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J. Neurosci. Res. 49:569-575 (1997)

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ABREVIATURAS

ARF,	Factor de ADP-ribosilación
BDNF,	Factor neurotrófico derivado de cerebro
BIM,	Bisindoleilmaleimida GF109203X
cAMP,	AMP cíclico
CAPK,	Proteína quinasa activada por ceramida
CAPP,	Proteína fosfatasa activada por ceramida
cdc42,	Proteína homóloga del gen de ciclo celular de levaduras 42
CNTF,	Factor ciliar neurotrófico
CRE,	Elemento sensible a cAMP
DAG,	Diacilglicerol
Elk-1,	Factor de transcripción similar a Ets
GAP,	Proteína activadora actividad GTPasa
GDNF,	Factor neurotrófico derivado de glía
GFs,	Factores de crecimiento
Grb2-sos,	Complejo proteico mediador de la activación de MAPK
hsp,	Proteína de choque térmico
ICER,	Represor temprano inducible por cAMP
IL,	Interleuquina
IP_3,	Inositol 1,4,5-trisfosfato
JAKs,	Quinasas del tipo Jano
JNK,	Proteína quinasa del extremo N terminal de c-Jun
LPA,	Acido lisofosfatídico
LPS,	Lipopolisacárido
MAPK,	Proteína quinasa activada por mitógenos
MEK,	Proteína quinasa de MAPK
mRNA,	Ácido ribonucléico mensajero
NGF,	Factor de crecimiento nervioso
PA,	Ácido fosfatídico
PAK,	Proteína quinasa activada por p21
PC,	Fosfatidilcolina
PE,	Fosfatidiletanolamina
PI,	Fosfatidilinositol
PMA,	4β-forbol 12β-miristato 13α-acetato
PKA,	Proteína quinasa dependiente de cAMP
PKC,	Proteína quinasa C
PLC,	Fosfolipasa C
PS,	Fosfatidilserina
Rho,	Proteína homologa de Ras
SAPK,	Proteína quinasa activada por estrés
SEK,	Proteína quinasa de SAPK
SF,	Esfingosina
SM, SMasa,	Esfingomielina, Esfingomielinasa
SNC, SNP,	Sistema nervioso central, Sistema nervioso periférico
src,	Proteína con dominio de homología SH
SRE,	Elemento de respuesta al suero
SRF,	Factor de respuesta al suero
STAT,	Proteínas con función de transducción de señales y factor de transcripción
TNF,	Factor de necrosis tumoral
Trk,	Receptor de neurotrofinas con actividad de tirosina quinasa
ZIP,	Proteína que interacciona con PKC ζ

1. SISTEMAS DE SEÑALIZACIÓN CELULAR

La regulación de la función celular depende de la transmisión de información del exterior al interior de la célula, lo que le permite responder de forma adecuada a un entorno cambiante y dinámico. Este proceso de comunicación intercelular se realiza a través de mensajeros químicos de diferente naturaleza. Los sistemas de transducción de señales están formados por un componente receptor y otro efector que induce la respuesta celular, pudiendo estos dos componentes funcionales formar o no parte de la misma entidad proteica. La unión del agonista al receptor promueve una respuesta celular mediada por la regulación del componente efector. Generalmente, los mensajeros intracelulares ejercen su efecto a través de fosforilación-desfosforilación de proteínas. Por último, los factores de transcripción permiten la regulación de la expresión génica.

A continuación se presentarán algunos de los componentes de sistemas de transducción de señales más directamente relacionados con el trabajo de investigación desarrollado en la presente tesis doctoral, destacando por ello, su papel en sistema nervioso y en particular en astrocitos y otras células gliales . Primero se describirán los distintos sistemas de generación de mensajeros lipídicos intracelulares a partir de glicerolípidos. Posteriormente se revisarán los actuales conocimientos sobre el papel de los esfingolípidos en la regulación celular a través de ceramidas. El estudio de los sistemas de fosforilación de proteínas incluirá una breve descripción aclaratoria de los sistemas de fosforilación regulados extracelularmente, MAPK y SAPK/JNK/p38, que se completará con el estudio de las características de la isoforma ζ de proteína quinasa C.

1.1 LOS LÍPIDOS COMO MENSAJEROS INTRACELULARES

Además de su función estructural, las membranas biológicas desempeñan un importante papel como fuente de generación de mensajeros intracelulares y extracelulares, que permiten la comunicación intercelular, la regulación del crecimiento, diferenciación, secreción, migración o apoptosis celular (Prescott, 1997). La investigación de los mecanismos de generación de mensajeros celulares de naturaleza lipídica constituye uno de los campos de la bioquímica de mayor avance en los últimos años. La acción de diferentes enzimas con actividad de fosfolipasa sobre glicerolípidos genera algunos de los mensajeros lipídicos mejor conocidos y caracterizados (Exton, 1997a;b; Leslie, 1997; Rhee y Bae, 1997). Recientemente se ha descrito la existencia de esfingolípidos con importantes efectos reguladores de la actividad celular, en lo

que se ha venido en llamar la ruta de la esfingomielinasa o de las ceramidas (Testi, 1996; Hannun, 1996; Spiegel et al., 1997).

FOSFOLIPASAS DE GLICEROLÍPIDOS

Fosfolipasa C

La actividad de la fosfolipasa C (PLC) sobre el fosfolípido genera diacilglicerol (DAG) y el grupo polar fosforilado, que pueden ser inositoles fosforilados, el más importante el inositol 1,4,5-trisfosfato (IP_3), para la fosfolipasa C específica de fosfatidilinositol (PI-PLC) o fosforilcolina en el caso de la fosfolipasa C específica de fosfatidilcolina (PC-PLC) (revisado por Exton 1997a; Rhee y Bae, 1997).

Fosfolipasa C de fosfatidilinositol

Los productos resultantes de la actividad de la PI-PLC actúan como mensajeros intracelulares: el DAG como activador de proteína quinasa C (PKC) y el IP_3 regulando la concentración de Ca^{2+} intracelular. Además, un descenso en los niveles de PIs en la membrana plasmática tiene otras consecuencias funcionales importantes en la célula, ya que algunos de estos fosfolípidos son el punto de unión a la membrana de proteínas con dominios de homología con la plecstrina, entre ellas la propia PI-PLC. Los PIs son además substrato de la enzima PI-3 quinasa, y así mismo cofactores de la fosfolipasa D específica de PC (Rhee y Bae, 1997). Existen diferentes formas de PI-PLC, las formas β se caracterizan por ser activadas fundamentalmente por receptores de siete segmentos transmembranares, a través de las subunidades α_q activas de proteínas G heterotriméricas (Chen et al., 1996), o por subunidades βγ. La activación de las formas PI-PLCγ tiene lugar mayoritariamente a través de receptores con actividad de tirosina quinasa (Rhee y Bae, 1997). El Ca^{2+} es necesario para la actividad de las distintas formas de PI-PLC, siendo las formas δ especialmente sensibles. La actividad de PI-PLC puede verse modulada también por ácido fosfatídico, ácido araquidónico e IP_3, lo que permite suponer la existencia de una activación de PI-PLC secundaria tras la activación de receptores acoplados a PLD, $cPLA_2$, o PI-3 quinasa (Rhee y Bae, 1997). La actividad de PI-PLC en astrocitos se activa por distintos agonistas como insulina, glutamato, bradiquinina o angiotensina II (Chen et al., 1996; Ruiz-Albusac et al., 1997; Tallant et al., 1997).

Fosfolipasa C de fosfatidilcolina

La PC-PLC esta implicada en la regulación del control de la proliferación celular (Díaz-Laviada et al., 1990; Bjorkoy et al., 1995; 1997; Cheng et al., 1997). El aumento de DAG intracelular resultante de la hidrólisis de PC es de larga duración y se mantiene elevado más allá del aumento transitorio generado por la PI-PLC (Asaoka et al., 1996; Nakamura, 1996). Este aumento de DAG, permite explicar la activación de PKC a largo plazo necesaria en la regulación del crecimiento y proliferación celular, siendo este proceso de especial importancia en las células gliales (Klein et al., 1995). El aumento en los niveles de DAG está en estrecha relación con la transformación celular (Laurentz et al., 1996). Como veremos más adelante la activación de PC-PLC se ha relacionado con la cascada de fosforilación de MAPK y con la PKC ζ (VanDijk et al., 1997a;b).

Fosfolipasa D

El substrato específico de la PLD es la PC, aunque de forma secundaria puede actuar sobre fosfatidiletanolamina (PE) o PI. La acción de la PLD sobre la PC genera ácido fosfatídico (PA) y colina libre (Fig. 1). El PA generado puede actuar directamente como mensajero intracelular, ser transformado en DAG por acción de una fosfatidato fosfohidrolasa, o ser metabolizado en ácido lisofosfatídico (revisado por Exton, 1997a;b). Alternativamente la PLD puede catalizar una reacción de transfosfatidilación por la que se transfiere el grupo fosfato a un alcohol primario, lo que se utiliza para la medida de su actividad.

La PLD, aunque mayoritaria en la membrana plasmática, se encuentra también en el núcleo, retículo endoplásmico, Golgi y citoplasma. La PLD se estimula por PI, Ca^{2+} y las proteínas G monoméricas ARF y Rho A (Exton, 1997a;b). Existen varias formas de PLD que se diferencian en su regulación, siendo la actividad PLD del Golgi más sensible a activación por ARF, mientras que la forma de membrana plasmática se activa fundamentalmente por Rho A y la forma citosólica por Ca^{2+}. La PKC activa la PLD, aunque no existen evidencias directas de una fosforilación directa sobre la propia PLD (Exton, 1997a;b).

La PLD cumple una importante función reguladora en la fisiología del sistema nervioso (Klein et al., 1995) y su actividad parece más importante, en términos generales, que la actividad de la PI-PLC en la regulación de la funcionalidad de los astrocitos (Jehan et al., 1995; Mangoura et al., 1995). En los astrocitos en cultivo primario existen importantes diferencias de regulación de PLD y en general en los sistemas de

señalización celular según el origen anatómico del sistema nervioso central (SNC) de los cultivos (Mangoura et al., 1995; Tallant y Higson, 1997).

El PA generado posee un efecto mitogénico que puede explicarse por su transformación en DAG, activador de PKC (Asoka et al., 1996; Nakamura, 1996), por su efecto modulador de Raf-1, componente de la cascada de MAPK (Ghosh y Bell, 1997) o por su transformación en LPA (Keller et al., 1997). En astrocitos algunos factores mitógenicos como trombina o endotelinas activan la PLD (Desagher, et al., 1997). Además, los niveles de PLD se regulan durante la diferenciación inducida por cAMP de las células de glioma C6 y en la apoptosis inducida por C2-ceramida en estas mismas células (Yoshimura et al., 1997).

Ceramidas y PLD

Las ceramidas generadas por acción de esfingomielinasas inhiben la actividad de PLD (Venable et al., 1996; Gomez-Muñoz et al., 1997) lo que potencia el efecto inhibidor del crecimiento de estos esfingolípidos (Hannun, 1996). La inhibición de PLD por ceramidas se debe al bloqueo de la translocación a membrana plasmática de Rho A, ARF, cdc42 y determinadas isoformas de PKC (Abousalham et al., 1997). Por otro lado, la esfingosina-1-fosfato, que se caracteriza por ejercer un efecto fundamentalmente mitogénico en las células (Spiegel et al., 1997), activa PLD (Desai et al., 1992). Existe por tanto una regulación cruzada entre las vías de señalización a través de esfingolípidos y glicerolípidos que aumentan la complejidad de los mecanismos de regulación celular (Gomez-Muñoz et al., 1997).

Fosfolipasa A_2

La acción de la PLA_2 libera el ácido graso situado en la posición *sn*-2 del fosfolípido, generando el lisofosfolípido correspondiente (revisado por Leslie, 1997) (Fig. 1). Existen distintas formas de PLA_2, una forma secretada, una forma independiente de Ca^{2+} y la forma citosólica, con características reguladoras específicas cada una de ellas. La actividad de la PLA_2 citosólica, $cPLA_2$, es especialmente importante en los astrocitos, interviniendo en la regulación del proceso de gliosis (Clemens et al., 1996). La liberación de ácido araquidónico por $cPLA_2$ regula la formación de diversos mensajeros lípidos. La completa activación de $cPLA_2$ implica primero su fosforilación por MAPK y posteriormente, gracias al Ca^{2+}, su unión a la membrana. Aunque la PKC incrementa la actividad de $cPLA_2$, esta activación parece ser debida a un efecto indirecto mediado por la activación previa de MAPK. TNFα, IL-1β, ATP y glutamato in-

crementan los niveles de $cPLA_2$ y aumentan su actividad en diferentes modelos celulares, entre ellos astrocitos y células de glioma C6 (Stella et al., 1997).

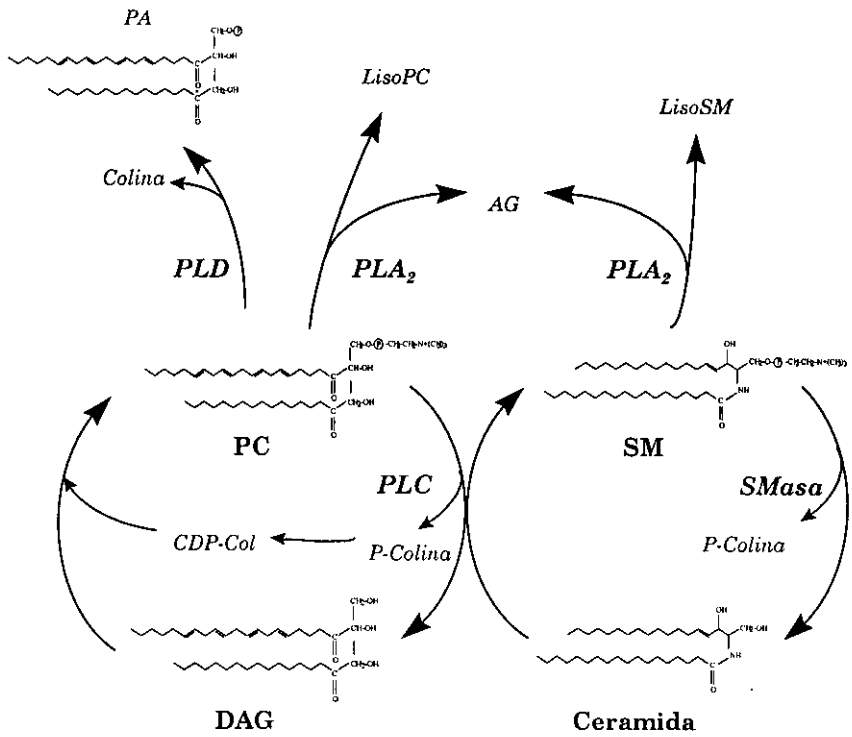

Fig. 1 Mensajeros lipídicos generados por acción de diferentes fosfolipasas sobre fosfatidilcolina (PC) y esfingomielina (SM).

LA RUTA DE LA ESFINGOMIELINASA-CERAMIDA

La identificación de los esfingolípidos como reguladores de los procesos de transducción de señales comenzó con la caracterización del efecto inhibidor de la esfingosina (SF) sobre la actividad de PKC dependiente de Ca^2. Actualmente se conoce la existencia de la denominada ruta de señalización de la esfingomielinasa (SMasa), también llamada de las ceramidas por ser este uno de sus productos con actividad biológica (revisado Hannun et al., 1996; Testi, 1996). La degradación de la esfingomielina (SM) conlleva la generación de ceramida (Figs. 1 y 2), que posteriormente puede ser transformada en SF, SF-1-P, o ceramida-1-fosfato que también pueden actuar como mensajeros celulares (Merrill et al., 1996; Spiegel et al., 1997). El aumento de ceramida es transitorio y la SM se resintetiza incorporando el grupo fosforilcolina procedente de PC a través de la sintasa de SM (Fig. 1).

TNFα, IL-1β, Fas, o la vitamina D3 fueron los primeros activadores identificados de este nuevo sistema de transducción de señales (Kim et al., 1991; Hannun et al., 1996). Posteriormente se ha descrito que el efecto de la vitamina D3 es debido a

un mecanismo autocrino de regulación mediado por la inducción de TNFα (Geilen et al., 1997). La lista de inductores de esta vía de señalización incluye, entre otros, agentes quimioterapéuticos, radiaciones ionizantes, NGF, interferón γ (Casaccia-Bonnefil et al., 1996a; Hannun et al., 1996). La SM está localizada de forma mayoritaria en la cara externa de la membrana plasmática, existiendo distintas fracciones ("pools") de SM implicadas en la señalización celular. Así la SM sensible a hidrólisis estimulada por TNFα está localizada en la cara interna de la membrana plasmática (Andrieu et al., 1996) y la SM sensible a NGF se localiza en determinados microdominios de membrana enriquecidos en caveolina (Bilderback et al., 1997).

Existen distintos tipos de SMasas con características de regulación diferentes. Se ha descrito la existencia de una actividad SMasa de membrana dependiente de Mg^{2+}, una SMasa citosólica independiente de Mg^{2+}, una SMasa denominada ácida, por poseer un pH óptimo de actuación ácido, que se localiza en los endosomas y una SMasa nuclear (Andrieu et al., 1994; Albi y Magni, 1997). Las distintas formas de SMasa parecen intervenir en diferentes mecanismos de señalización celular, lo que puede explicar la existencia de respuestas celulares opuestas activadas por la ruta de la SMasa. En el caso del receptor de 55 kDa del TNFα, existen dos dominios citosólicos que activan de forma independiente la SMasa ácida y la SMasa neutra. La activación de la SMasa ácida está precedida por la activación de PC-PLC. El aumento en los niveles intracelulares de ceramidas desencadenado por este mecanismo sería responsable de la activación del factor nuclear de transcripción NF-κB (Schütze et al., 1992; Wiegmann et al., 1994). El otro dominio citosólico interacciona con una proteína adaptadora FAN y activa SMasa neutra (Adam-Klages et al., 1996). La generación de ceramidas por esta rama de señalización puede activar la cascada de MAPK y de este modo inducir la activación de $cPLA_2$ (Adam-Klages et al., 1996). Sin embargo, en otros modelos, la activación de $cPLA_2$ es un requisito previo necesario para la generación de ceramidas estimulada por TNFα (Jayadev et al., 1997).

Las ceramidas pueden activar una proteína fosfatasa de tipo 2A, CAPP, que fue identificada en células de glioblastoma T9 y que puede regular el estado de fosforilación de c-Jun (Gonzalez et al., 1996). Además, existe una proteína quinasa activada por ceramida (CAPK) que recientemente se ha identificado como la quinasa supresora de Ras (Zhang et al., 1997a), y que puede fosforilar y activar Raf-1 (Yao et al., 1995).

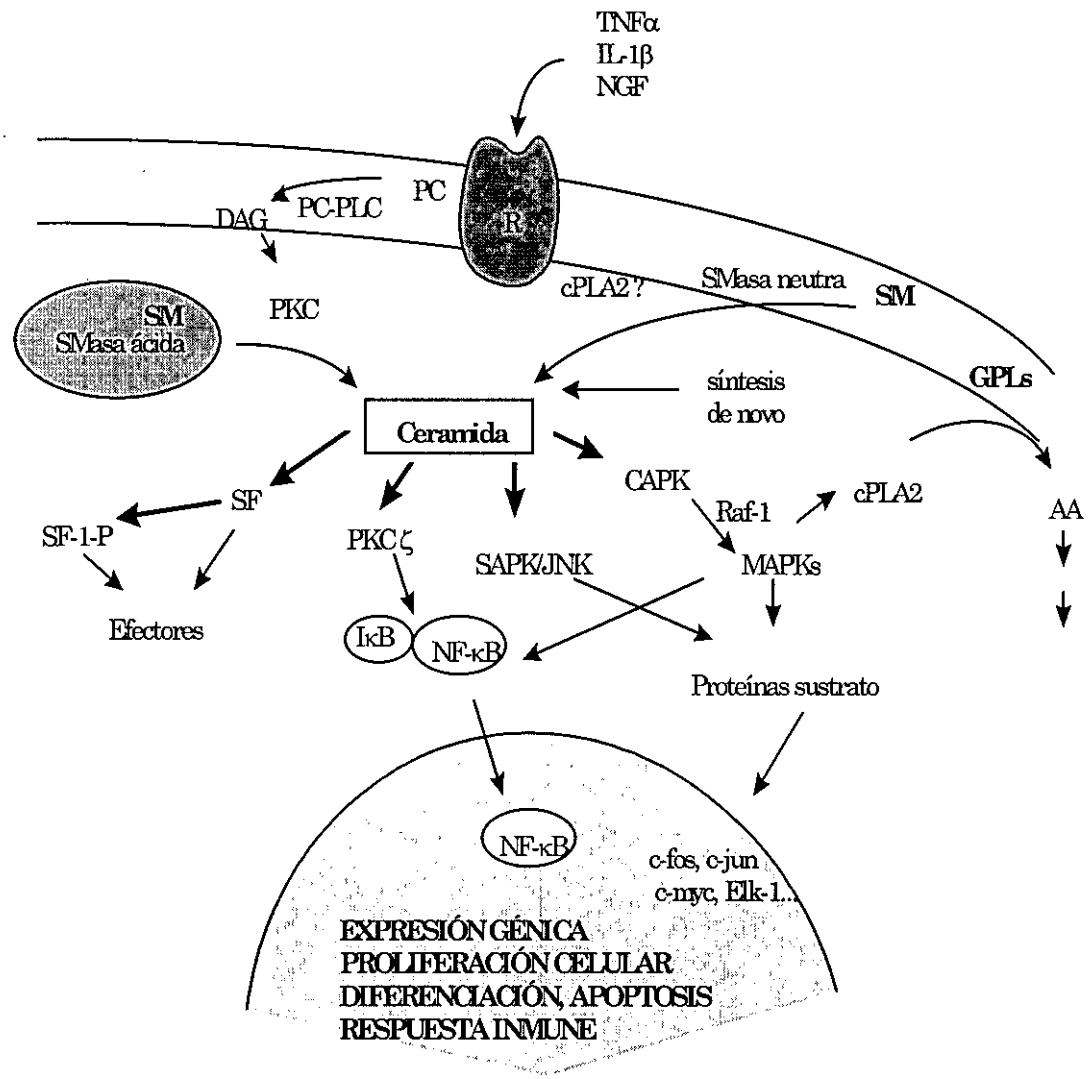

Fig. 2 Esquema de la ruta de señalización celular de las ceramidas

La estimulación por TNFα y el aumento intracelular de ceramidas incrementan la actividad MAPK (Sasaki et al., 1995; Modur et al., 1996), sin embargo, parece que es más importante la activación de esta quinasa por SF-1-P y factores de crecimiento (Coroneos et al., 1996; Pyne et al., 1996). Las ceramidas son potentes activadores de SAPK/JNK (Westwick et al., 1995), lo que puede explicar su efecto apoptótico (Verheij et al., 1996). El aumento en los niveles de DAG intracelular inhibe la apoptosis, lo que se explica por su efecto inhibidor en la generación de ceramida (Laurenz et al., 1996). Existe por tanto un complejo mecanismo de regulación de la proliferación y muerte celular en función del contexto fisiológico en el que se generan los distintos tipos de mediadores lipídicos.

Efectos biológicos de las ceramidas

Las ceramidas se han relacionado con la inhibición del crecimiento, la diferenciación celular y con la apoptosis estimulada por citoquinas inflamatorias y Fas (Hannun, 1996). Además de ejercer un importante control de los procesos del crecimiento celular y axonal, las ceramidas regulan la expresión génica a través de factores de transcripción como NF-κB o AP-1, procesos de endocitosis y canales de K^+ (Hannun et al., 1996). Los efectos antiproliferativos de las ceramidas pueden estar mediados por la disminución de los niveles de *c-myc* a través de CAPP. La elevación de los niveles de ceramida puede detener las células en las etapas Go/G1 del ciclo celular, manteniendo la proteína de retinoblastoma pRb en un estado hipofosforilado.

En sistema nervioso, las ceramidas pueden inducir la muerte celular en oligodendrocitos, mientras que los astrocitos son resistentes a este fenómeno (Casaccia-Bonnefil et al., 1996a;b). En cultivos neuronales se ha observado que las ceramidas pueden inducir o impedir la apoptosis, según el origen o momento de desarrollo (Wiesner y Dawson, 1996; Scharwz y Futerman, 1997). Sin embargo, parece que la apoptosis inducida por ciertos activadores de la vía de las ceramidas como Fas, puede ser independiente de la generación de ceramidas (Watts et al., 1997). En cualquier caso, existen distintas fracciones de SM implicados en la señalización celular, y no todas son capaces de inducir apoptosis en la célula (Zhang et al., 1997b).

La mayor parte del conocimiento del papel de los esfingolípidos en la señalización celular se fundamenta en la generación de ceramida concomitante a la degradación de SM. Sin embargo, la degradación de los niveles de ceramida por la ceramidasa, o la regulación de su síntesis son también cruciales en el control de los niveles intracelulares de ceramida (Bose et al., 1995; Biewlaska et al., 1996; Nikolova-Harakashian et al., 1997). La diferente regulación que ejercen citoquinas y factores

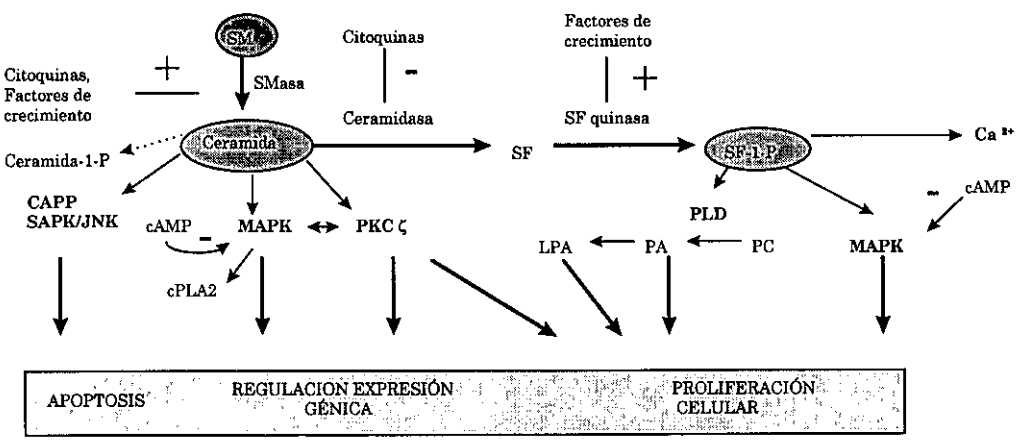

Fig. 3 Esquema de la regulación que sobre la ruta de las ceramidas ejercen citoquinas por-inflamatorias y factores de crecimiento

de crecimiento sobre SMasa, ceramidasa y quinasa de SF puede determinar el balance entre los niveles de ceramidas y SF/SF-1-P lo que permite controlar el balance muerte celular/diferenciación o proliferación a través de la activación de distintas cascadas de fosforilación (Fig.3) (Coroneos et al., 1995; 1996; Cuvillier et al., 1996; Pyne et al., 1997).

1.2 SISTEMAS DE FOSFORILACIÓN DE PROTEÍNAS

La fosforilación y desfosforilación de proteínas por proteína quinasas y fosfatasas constituye uno de los procesos reguladores más comunes en la célula. Su organización en cascadas de fosforilación permite una amplificación de la señal, una gran sensibilidad en la respuesta, la integración de la información procedente de diferentes estímulos así como la aceleración de la respuesta celular. En eucariotas, la fosforilación de proteínas con fines reguladores se lleva a cabo fundamentalmente en serina/treonina o tirosina, resultando excepcional la posibilidad de fosforilación de ambos tipos de aminoácidos ("actividad dual"). A continuación se hace una breve descripción de diferentes cascadas de fosforilación reguladas extracelularmente: MAPK, SAPK/JNK y p38. Además se tratarán de forma específica las características reguladoras de la isoforma ζ de la proteína quinasa C.

SISTEMAS DE FOSFORILACIÓN EN CASCADA REGULADOS EXTRACELULARMENTE

Los sistemas de fosforilación de proteínas regulados por estímulos extracelulares incluyen al menos tres grandes grupos de señalización: MAPKs , SAPK/JNKs y p38 (Fig. 4). Los primeros se caracterizan por ser activados fundamentalmente por agentes mitogénicos que actúan a través de receptores con actividad de tirosina quinasa (Denhart, 1996). Los segundos se activan por estrés celular y citoquinas proinflamatorias a través de receptores que carecen de actividad de tirosina quinasa (Kyriakis y Avruch, 1996). Algunas de las serina/treonina quinasas que forman parte de estos sistemas de fosforilación se caracterizan por precisar de una doble fosforilación en tirosina y treonina, para alcanzar su máximo grado de activación, lo que permite un control muy preciso en su regulación (Denhardt, 1996; Kyriakis y Avruch, 1996).

MAP quinasas

El sistema de fosforilación por la cascada de MAPK incluye la activación sucesiva de Ras/Raf-1/MEK y MAPK (Fig. 4). Esta vía de transducción de señales constituye uno de los principales sistemas de control de proliferación celular tanto en células gliales como de diferente origen (Cazaubon et al., 1997; Van Brocklyn et al., 1997). El NGF se encuentra entre la gran variedad de mensajeros con receptores de actividad de tirosina quinasa que utilizan este sistema de transducción de señales (Segal y Greenberg, 1996). En general, la activación de receptores con actividad de tirosina quinasa por sus agonistas induce su autofosforilación y dimerización. Los residuos de tirosina fosforilados son reconocidos por una serie de proteínas que contienen una estructura de homología en dominios denominados SH2 ("src homology 2"), estando el reconocimiento de tirosinas fosforiladas condicionado por el contexto de aminoácidos en el que se encuentran. El receptor fosforilado une directamente o a través de proteínas adaptadoras, el complejo citosólico preexistente Grb2-Sos (revisado por Denhart, 1996). Sos es una proteína activadora del intercambio de GDP por GTP, lo que permite la activación de Ras localizada en la membrana plasmática y que pertenece a la superfamilia de las proteínas G monoméricas. En la regulación de Ras además de proteínas que facilitan el intercambio GTP-GDP, participan proteínas activadoras de su actividad GTPasa, GAP, importantes para la integración de la información de señales procedentes de Ras y Rho (Denhart, 1996). Los receptores con siete segmentos transmembranares también pueden inducir respuestas mitogénicas a través de la cascada de MAPKs (revisado por Post y Brown, 1996). Así, la activación de PI-PLC vía Gq/11 genera DAG que junto con el aumento de Ca^{2+} intracelular promovido por IP_3 permiten la plena activación de determinadas PKCs, lo que puede desembocar en la activación de la cascada de MAPK. La activación de Gi, además de inhibir la adenilato ciclasa, libera subunidades $\beta\gamma$ que pueden activar Ras a través de tirosinas quinasa citosólicas como src.

Raf-1

La proteína Raf-1 es una serina/treonina quinasa activada por la interacción con Ras, para la que posee dos posibles sitios de unión (revisado por Morrison y Cutler, 1997). La activación de Raf-1 depende de su translocación a la membrana que es dirigida por la proteína Ras activa (Stokoe et al., 1994), de modo que su translocación, independientemente del grado de fosforilación, determina su activación (Stokoe y McCormick, 1997). La proteína Raf-1 esta asociada de forma constitutiva con las pro-

teínas hsp 90, hsp50 y 14-3-3. La interacción con 14-3-3 favorece el mantenimiento de Raf-1 en su forma inactiva dado que interfiere la unión de Ras a través del segundo sitio (Clark et al., 1997). La proteína 14-3-3 es una proteína que une residuos de serina fosforilados posible componente de una familia de proteínas aún desconocida, análogas a las que poseen dominios SH2 que unen tirosinas fosforiladas, y que permitirían la formación de complejos multiproteicos (Muslin et al., 1996).

Raf-1 se puede activar por interacción específica con ceramidas, lo que permite establecer una relación directa entre la ruta de señalización de las ceramidas y la activación de MAPK (Huwiler et al., 1996). Además, el PA, producto de la acción de PLD, también puede activar Raf-1 (Ghosh y Bell, 1997). Del estudio de la secuencia de Raf-1 y del análisis funcional de sus distintos dominios, se ha postulado la existencia de un elevado grado de analogía con la familia de PKC (Ghosh y Bell, 1997).

La regulación de Raf-1 por los sistemas de fosforilación es compleja y dista de ser bien comprendida. Por un lado la fosforilación en tirosina por src parece ser necesaria para su plena activación junto con la interacción con Ras-GTP (Marais et al., 1995). Sin embargo, existen sistemas en los que la activación de Raf-1 no va acompañada de su fosforilación en tirosina (Wartmann et al., 1997). La fosforilación de Raf-1 en treonina o serina por CAPK o por PKC promueve también su activación (Yao et al., 1995). La isoforma ζ de PKC atípica puede activarse por Ras (Diaz-Meco et al., 1994) lo que podría explicar la activación de MEK/MAPK por PKC ζ (Berra et al., 1995), tal vez a través de la activación directa de PKC ζ sobre Raf-1 (VanDijck et al., 1997a). Por otro lado, la fosforilación de una serina específica por PKA inhibe Raf-1, lo que indica la existencia de regulación cruzada entre diferentes sistemas de transducción de señales (Hafner et al., 1994). En cualquier caso, el grado de fosforilación es fundamental en la regulación de la actividad de Raf-1, y su hiperfosforilación conduce a una menor afinidad por la membrana plasmática y constituye uno de los mecanismos de inactivación (Wartmann et al., 1997).

La activación de Raf-1 por fosforilación permite activar las proteína MEKs de especificidad dual (MEK1 y MEK2). La fosforilación de MEKs por Raf-1 está dirigida por la existencia de dominios SH3 ricos en prolina. Posteriormente MEK activa las proteínas quinasas MAPK1 y MAPK2. Estas enzimas pueden translocarse al núcleo y por fosforilación activar substratos como proteínas asociadas a microtubulos, proteínas quinasas ribosomales, $cPLA_2$ y factores de transcripción como Elk-1 y c-myc, lo que permite el control de importantes procesos celulares como la proliferación, la organización del citoesqueleto y la regulación de la expresión génica.

Regulación de la actividad MAPK en astrocitos

La activación de la cascada de MAPKs es esencial en la regulación de la proliferación de los astrocitos. Factores mitogénicos como endotelinas, FGF, PDGF, los gangliósidos o el péptido P estimulan la actividad de MAPK de forma sostenida (Kurino et al., 1996; Lazarini et al., 1996; Luo et al., 1996; Cazaubon et al., 1997; VanBrocklyn et al., 1997). El aumento en los niveles intracelulares de cAMP inhibe la proliferación de astrocitos y activa la diferenciación hacia un fenotipo astrocitario en las células de glioma C6 (Chen et al., 1996; Anciaux et al., 1997), estando la inhibición de la proliferación directamente relacionada con la inhibición de la actividad MAPK (Kurino et al., 1996; Prins et al., 1996). La inhibición de MAPK por el aumento intracelular de cAMP se observa también en la activación de MAPK inducida por el lipopolisacárido (LPS) bacteriano (Willis y Nisen, 1996).

La activación de MAPK por endotelinas en astrocitos ocurre por un doble mecanismo: el clásico de fosforilación en tirosina del adaptador Shc, asociación a Grb2 y activación de Raf-1 (Lazarini et al., 1996) y por un mecanismo dependiente de Rho y proteínas de matriz extracelular (Cazaubon et al., 1997).

La actividad MAPK es importante también en la regulación del espacio sináptico, condicionado por la regulación del volumen celular de los astrocitos (Porter y McCarthy, 1997). La reducción del volumen celular en los astrocitos en respuesta a un estímulo hipoosmótico tiene lugar a través de un aumento intracelular de Ca^{2+}, vía IP_3 y la activación de MAPK dependiente de PI-3 quinasa (Schliess et al., 1996).

SAPK/JNK y p38

De modo semejante a la cascada de fosforilación Ras/Raf/MAPK activada por factores mitogénicos, se ha descrito la existencia de sistemas de transducción activados por estrés celular (rayos UV, rayos X) y citoquinas inflamatorias (revisado por Kyriakis y Avruch, 1996). Estos sistemas de transducción de señales generan en la célula fundamentalmente respuestas inhibitorias del crecimiento, diferenciación o muerte celular (Coroneos et al., 1996; Hannun, 1996; Brenner et al., 1997). Los receptores de TNFα o IL-1β, carecen de actividad de tirosina quinasa y su activación va asociada a la activación de proteínas tirosina quinasas citosólicas como JAKs (quinasas del tipo Jano) que se autofosforilan y fosforilan el receptor permitiendo la unión de proteínas adaptadoras (Baker y Reddy, 1996). La activación de SAPK/JNK está precedida por la hidrólisis de SM y generación de ceramida, y las ceramidas son capaces de activar SAPK/JNK de forma específica (Westwick et al., 1995), lo que

permite atribuir un papel a este mensajero en su regulación. En efecto, la apoptosis inducida por citoquinas inflamatorias y estrés celular puede estar mediada por la activación de SAPK/JNK a través de ceramida (Verheij, et al., 1996). En concreto, en cultivos primarios de astrocitos, la activación de SAPK por TNFα coincide con su activación por la adición de SMasa exógena o ceramidas permeables, sin embargo, la cinética de activación de SAPK por SMasa y ceramidas no coincide con la inducida por el TNFα (Zhang et al., 1996).

La activación de SAPKs por los receptores de membrana se lleva a cabo fundamentalmente por las proteínas G monoméricas Rac1 y Cdc42 de la familia Rho, siendo la activación de SAPK independiente de la acción directa de Ras (Kyriakis et al., 1994). Estas proteínas activan MEKK1 a través de las proteínas PAK, que inhiben la actividad GTPásica de las proteínas Rho. La proteína MEKK1, situada a nivel de Raf-1 en la cascada de fosforilación mitogénica (Fig. 4), es el activador de SEK1/MKK4 y también de MEK (Kyriakis y Avruch, 1996). La proteína SEK1/MKK4 es la proteína homóloga de MEK responsable de la activación específica de SAPK e incapaz de activar MAPK. Las SAPKs, también se denominan quinasas del extremo N terminal de c-Jun (JNKs) debido a que este factor de transcripción es uno de sus substratos, junto a otros como ATF-2 y Elk-1 (Kyriakis et al., 1994). Recientemente, se ha demostrado que este sistema de transducción de señales es fundamental en la regulación del proceso de gliogénesis y diferenciación de los astrocitos a partir de células progenitoras corticales (Bonni et al., 1997).

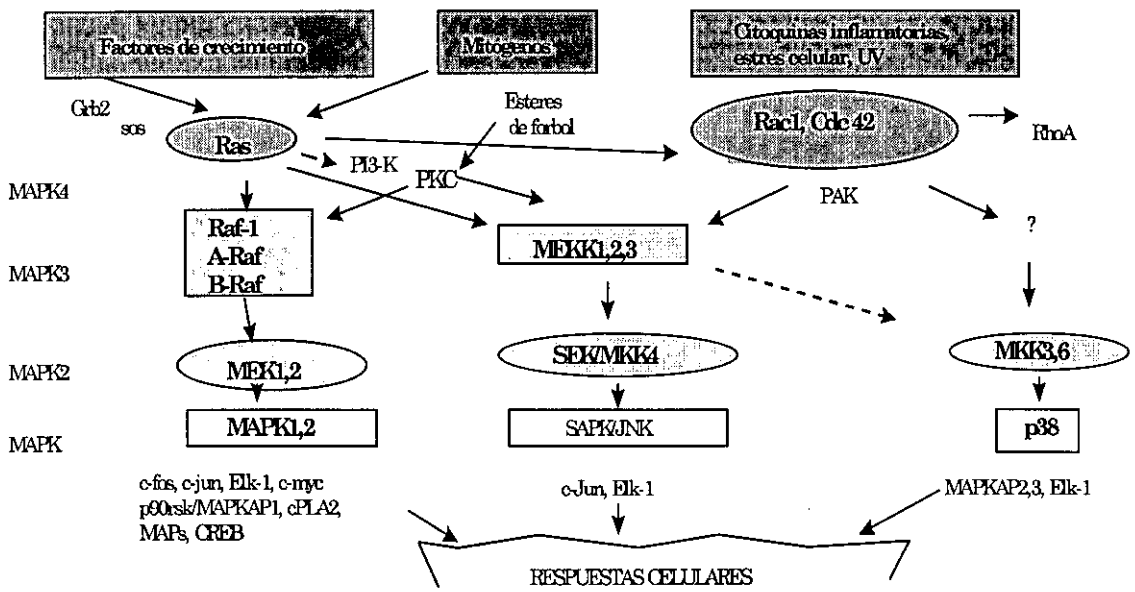

Fig. 4 Esquema de las principales cascadas de fosforilación reguladas extracelularmente

Además del sistema SAPK/JNK existe en células de mamíferos una serie de proteínas homólogas al sistema de señalización de estrés celular osmótico en levaduras (HOG1)(Takekawa et al., 1997), denominado p38 (Fig. 4). Esta cascada de fosforilación activada por estrés celular y citoquinas inflamatorias es paralela a SAPK e implica a las quinasas MKK3/MKK6 (Kyriakis y Avruch, 1996).

La activación de Ras por agentes mitogénicos puede activar de forma secundaria SAPK a través de la activación de MEKK1 o directamente de Rac-cdc42 (Kyriakis y Avruch, 1996). La existencia de puntos de regulación cruzada entre las distintas cascadas de fosforilación descritas, permite su coordinación en diferentes sistemas celulares como los astrocitos (Korzus et al., 1997).

LA ISOFORMA ζ DE PROTEÍNA QUINASA C

El término PKC engloba una familia de proteínas quinasas caracterizadas inicialmente por su actividad dependiente de Ca^{2+} y DAG (Inoue et al., 1977). Actualmente se han descrito al menos 12 isoformas de PKC que se agrupan en función de su estructura y características funcionales en tres categorías. Las PKCs convencionales α, βI, βII y γ, cPKCs, que precisan para su actividad de Ca^{2+}, DAG (o sus análogos los ésteres de forbol) y fosfatidilserina (PS). Las PKCs denominadas nuevas δ, ε, θ, η/L (ratón/humano), nPKCs, tienen los mismos requerimientos de cofactores excepto que no precisan de Ca^{2+}. Por último la categoría de las PKCs atípicas λ/ι (ratón/humano) y ζ, aPKCs, únicamente requieren para su actividad de PS, y son independientes del Ca^{2+} o del DAG (revisado por Jaken, 1996; Hoffman, 1997). El Ca^{2+} se une al denominado dominio C2 y el DAG y sus análogos al dominio C1. Las distintas isoformas de PKC se expresan en astrocitos y en células de glioma C6 (Ballestas y Benveniste, 1995; Chen et al., 1995; 1996), con la excepción de la forma cPKC γ (Roisin y Deschepper, 1995). Las características intrínsecas reguladoras de cada grupo de PKC determinan que cada tipo sea activado preferentemente por determinados sistemas de transducción de señales. Así, las nPKCs parecen ser activadas fundamentalmente por la generación de DAG vía PLD, mientras que las cPKCs precisan para su completa activación del aumento de Ca^{2+} intracelular que acompaña a la hidrólisis de fosfoinosítidos vía PI-PLC (Ha y Exton, 1993).

La activación de PKC va acompañada de su translocación desde la fracción citosólica a la bicapa lipídica, donde encontrará la PS necesaria para su actividad enzimática. La PKC después de una activación persistente sufre una proteolisis parcial,

seguido de un proceso de degradación posterior que conduce a una disminución de los niveles de enzima, denominado "downregulation" (Jaken, 1996; Hoffman, 1997).

PKC atípica

El grupo de las PKCs atípicas incluye dos isoformas λ/ι y ζ. La ausencia del dominio C2, y el poseer únicamente una estructura en "dedo de zinc" en C1, explica su independencia de Ca^{2+} y DAG (Wooten et al., 1997). La PKC ζ se puede activar por el producto de la PI-3 quinasa (Nakanishi et al., 1993), y también por PA (Limatola et al., 1994). La PKC ζ es por sus características estructurales resistente a la activación y "downregulation" por ésteres de forbol, como ocurre en los astrocitos (Ballestas y Benveniste, 1995; Chen et al., 1995), y esta enzima podría estar implicada en el control de la diferenciación astrocitaria (Yoshimura et al., 1997). Resulta interesante la observación de que la activación de MAPK por endotelinas sea independiente de la actividad de PKCs sensibles a "downregulation" por ésteres de forbol (Tournier et al., 1994). Además, la PKC ζ se encuentra implicada en la diferenciación de células PC12 por NGF (Coleman y Wooten, 1994), el proceso de potenciación a largo plazo, "long-term potentiation" (Hrabetova y Sacktor, 1996), y el control de la proliferación celular (Berra et al., 1993; Lehrich y Forrest, 1994). La funcionalidad de la PKC ζ está íntimamente asociada a su localización subcelular (Wooten et al., 1996), y el proceso de diferenciación celular precisa de la translocación de PKC ζ a la matriz nuclear (Wooten et al., 1997). La localización subcelular de PKC ζ puede estar regulada por su interacción con proteínas específicas como ZIP ("zeta interacting protein") o PICK (Puls et al., 1997). La interacción con este tipo de proteínas puede ser importante para aumentar la especificidad de la interacción de aPKCs con los lípidos que actúan como cofactores (Diaz-Meco et al., 1996). La translocación nuclear de PKC ζ puede venir condicionada también por la presencia de mensajeros lipídicos, como el PA generado vía PLD nuclear, o por la existencia de secuencias específicas de señalización nuclear (Wooten et al., 1997).

Sistemas de transducción de señales y PKC ζ

La PKC ζ actúa de forma cooperativa con Ras y podría coordinar los efectos de PC-PLC y PI-3 quinasa en el control de la proliferación celular (Nakanishi et al., 1993; Diaz-Meco et al., 1994), actuando en un nivel anterior a MAPK (Berra et al., 1995). La PKC ζ activada por la PC-PLC es un activador de Raf-1, lo que explicaría la

existencia de un proceso de activación de MAPK independiente de Ras (van Dijk et al., 1997a).

El modelo de Moscat y colaboradores propone que la PKC ζ es un importante regulador de la mitogénesis e inhibidor de la apoptosis (Berra et al., 1997). Sin embargo, la PKC ζ revierte el fenotipo transformado inducido por la expresión de v-Raf en células 3T3 (Kieser et al., 1996), y su sobrexpresión no tiene consecuencias mitogénicas en estas mismas células (Crespo et al., 1995; Montaner et al., 1995).

La PKC ζ parece estar implicada en el mecanismo de transducción de señales del TNFα e IL-1β, a través de la activación de la SMasa ácida previa activación de la PC-PLC (Lozano et al., 1994; Carlson y Hart, 1996). El producto de acción de la SMasa, la ceramida, es en efecto un activador de PKC ζ. Concentraciones bajas de ceramida activan PKC ζ, pero niveles mayores revierten esta activación. Por último el ácido araquidónico compite con la ceramida, inhibiendo así la activación que esta ejerce sobre PKC ζ (Müller et al., 1995). La PKC ζ puede servir por tanto como mecanismo de integración de señales mitogénicas o de inhibición del crecimiento.

1.3 REGULACIÓN DE LA EXPRESIÓN GÉNICA

La regulación de la expresión génica esta basada en su activación o inhibición por los denominados factores de transcripción y puede considerarse la última etapa del proceso de transmisión de información que comienza en la membrana plasmática. La propia estructura del DNA en asociación con histonas y las modificaciones químicas de éstas permiten mecanismos de control adicionales. Para ejercer su acción, los factores de transcripción deben localizarse en el núcleo e interaccionar con el DNA. La regulación a través de factores de transcripción puede ejercerse a través de la regulación de su translocación al núcleo o por la inducción de su síntesis por señales extracelulares. El estado de fosforilación es esencial en la regulación de factores de transcripción, favoreciendo cambios conformacionales que permiten exponer secuencias de señalización nuclear, modificando el estado de oligomerización de la proteína, regulando la interacción proteína-DNA o regulando su interacción con el sistema celular de transcripción (Hill y Treisman, 1995). A continuación se describe el sistema de regulación de tres tipos fundamentales de factores de transcripción.

El factor de transcripción NF-κB está localizado en el citosol en asociación con la subunidad IκB que ejerce un efecto inhibidor que impide su translocación al núcleo. Citoquinas inflamatorias, estrés celular, LPS o infección viral, entre otros, son activadores de NF-κB (Kohler et al., 1997). La fosforilación de IκB permite su degrada-

ción, y la consiguiente translocación de NF-κB al núcleo donde ejerce su efecto regulador de la transcripción (O'Neill y Kaltschmidt, 1997). La transcripción de numerosos genes está regulada por el factor NF-κB, y es esencial en la funcionalidad glial y neuronal (O'Neill y Kaltschmidt, 1997). La identificación de la proteína quinasa responsable de la fosforilación y degradación de IκB, es actualmente uno de los objetivos para el conocimiento de los mecanismos implicados en la activación de este factor de transcripción (Israël, 1997).

Otro tipo de factores de transcripción esta representado por c-Fos. Esta proteína pertenece al grupo de los genes de expresión temprana. La activación celular induce la expresión del gen c-fos. La proteína c-Fos sintetizada constituye homodímeros, o heterodímeros con c-Jun, complejos AP-1, que regulan la expresión génica. El promotor de c-fos posee un elemento denominado SRE ("serum response element") que es un sitio de unión de factores de transcripción activados por el suero. La activación de SRE requiere de la unión del factor SRF y de Elk-1. Elk-1 es activado por fosforilación a través de la cascada de las MAPK, de modo que la translocación nuclear de MAPK permite su fosforilación y activación de Elk-1 (Denhart, 1996).

Las proteínas STATs, son factores de transcripción activados por su fosforilación en la membrana plasmática a través de tirosina quinasas JAKs asociadas a receptores de la familia de las citoquinas. La fosforilación permite la dimerización de STATs y su translocación al núcleo (Darnell, 1997). Al igual que ocurre con el factor AP-1, la distinta composición de los oligómeros puede condicionar la especificidad en la interacción complejo proteico-secuencia de DNA diana.

Tan importante como la activación de los factores de transcripción es su vuelta al estado basal. Las proteínas fosfatasas revierten la activación de los factores de transcripción activados por fosforilación. La inducción de la síntesis de factores inhibitorios, como IκB o de ICER, que compite en la unión de factores a CRE ("cAMP responsive element"), es otro modo de inactivación de la expresión génica. La comunicación entre los sistemas de transducción de señales y los factores de transcripción no es unidireccional, y estos últimos también pueden regular la actividad de los primeros. Así, la activación de NF-κB puede activar la PKA de forma independiente de los niveles de cAMP a través de la disociación de complejos NF-κB-IκB-PKA que mantienen la PKA en estado inactivo (Zhong et al., 1997).

Un mismo estímulo extracelular puede regular diferentes factores de transcripción, y las células reciben multitud de estímulos de forma simultánea. Esto sumado a los efectos cruzados de regulación entre distintas rutas de señalización celu-

lar, permite imaginar una situación en la que la regulación de la expresión génica está modulada por un delicado balance entre numerosos sistemas de control.

2. EL FACTOR DE CRECIMIENTO NERVIOSO

El factor de crecimiento nervioso (NGF) fue el primer factor de crecimiento, supervivencia y diferenciación neuronal caracterizado y con el que se constituyó la familia de las neurotrofinas (Levi-Montalcini, 1987). El NGF fue descubierto como el factor difusible responsable de la producción de un halo de fibras nerviosas en un explante de ganglios simpáticos y sensoriales de embrión de pollo (Levi-Montacini y Hamburger, 1953). La teoría neurotrófica sostiene que las neuronas compiten entre sí por una cantidad limitada de neurotrofinas proporcionadas por el tejido inervado. Las neurotrofinas juegan un papel esencial en la regulación del desarrollo del sistema nervioso, la plasticidad sináptica del sistema nervioso adulto y en el mantenimiento de su integridad estructural (Hefti, 1994). La ausencia de NGF, o una disminución local en sus niveles, impide el desarrollo neuronal normal y explica la apoptosis fisiológica durante el periodo neonatal. Además el propio NGF participa en la activación de procesos apoptóticos mediados por el receptor $p75^{NTR}$ en neuronas deficientes en trkA (Dechant y Barde, 1997). La familia de las neurotrofinas incluye además el factor neurotrófico derivado del cerebro (BDNF) y las neurotrofinas NT-3, NT4/5 y NT-6 (Varon et al., 1995; Lewin y Barde, 1996). Cada tipo de neurotrofina ejerce su efecto de forma preferente en determinadas poblaciones neuronales y en momentos específicos del desarrollo. Existen otras proteínas con efectos neurotróficos como el factor neurotrófico derivado de la glía (GDNF) o el factor ciliar neurotrófico (CNTF) con características diferenciales que las separan de la familia de las neurotrofinas.

Además de los efectos "convencionales" del NGF en el sistema nervioso, este factor, desempeña un papel crucial en la regulación de las relaciones entre el sistema nervioso, inmune y neuroendocrino, existiendo una comunicación bidireccional entre sistema nervioso y sistema inmune (Levi-Montalcini et al., 1996; Merrill y Benveniste, 1996). El NGF ejerce un efecto proliferativo sobre linfocitos T y B, constituye un factor autocrino de supervivencia de los linfocitos B memoria y estimula la producción de inmunoglobulinas (Torcia et al., 1996). Además, actúa como factor quimiotáctico regulando la funcionalidad de microglía y macrófagos y es un mediador de la respuesta de fase aguda (Gilad y Gilad, 1995; Elkabes et al., 1996). La existencia de elevados niveles de NGF en algunas situaciones patológicas como esclerosis múltiple o encefa-

litis alérgica experimental (Bonini et al., 1996; DeSimone et al., 1996), evidencian la importancia de la interconexión entre el sistema inmune y el sistema nervioso.

2.1 EL GEN DEL NGF

El gen del NGF se ha clonado en diferentes organismos, estando situado en el hombre en la región 1p13 del cromosoma 1 (Scott et al., 1983; Carrier et al., 1996). El gen de NGF está formado por cuatro exones (Fig. 5) y su transcripción da lugar a la síntesis de cuatro transcritos (A, B, C y D), debidos a la existencia de un doble sitio de iniciación de la transcripción y por procesamiento diferencial ("splicing") del pre-mRNA, siendo el principal transcrito de 1.35 kb (Selby et al., 1987).

La región de 5 kb situada delante del primer exón posee los elementos necesarios responsables de la regulación específica de tejido del gen (Alexander et al., 1989). El promotor principal del gen de NGF posee una caja TATA y CCAAT, elementos fundamentales para la unión de la RNA polimerasa y sus cofactores. En rata, las cajas TATA están situadas en la posición -43 y -23, separadas por 15 nucleótidos. Las cajas CCAAT, asimilables a cajas CAAT, están situadas en las posiciones -379 y -546. Existen dos elementos represores en las posiciones -500 y -120 y también dos secuencias activadoras entre -120 y -39 (D'Mello y Heinrich, 1991a; Cartwright et al., 1992). En la región +33 a +50, en el límite del exón 1b con el intrón siguiente existe un elemento regulador AP-1 (Fig. 5) (Hengerer et al., 1990), y en la posición -669 se encuentra un posible sitio de regulación por NF-κB (Jehan et al., 1993). La existencia de una secuencia AP-1 permite explicar la inducción de NGF por estímulos activadores de c-fos y c-jun como el PMA, suero, cAMP y daño cerebral (Jehan et al., 1993; Onteniente et al., 1994; Colangelo et al., 1996). Sin embargo, existen otros elementos reguladores

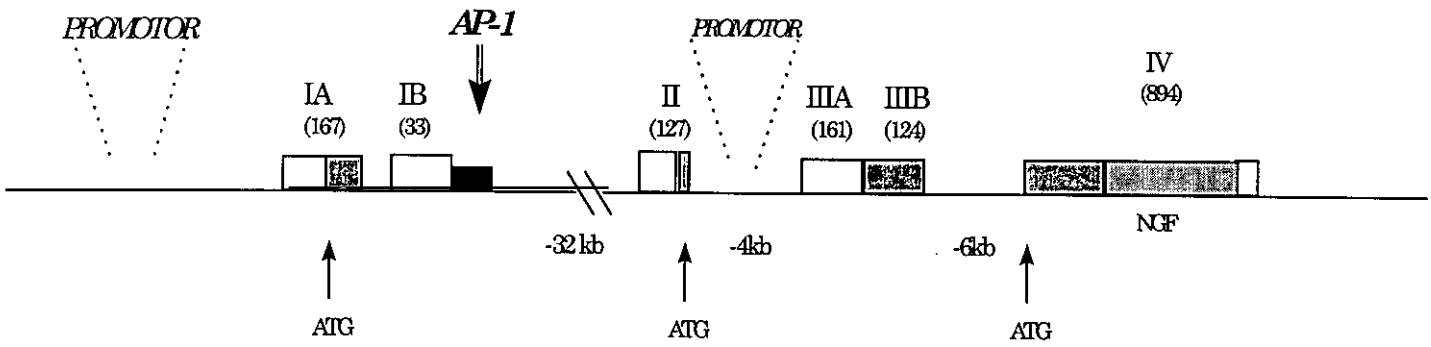

Fig. 5 Representación esquemática del gen de NGF en rata. Los rectangulos representan los exones: en blanco son regiones no traducidas, rallados codifican partes del precursor de NGF y en gris se muestra la región codificante de la proteína activa.

aún no caracterizados que explicarían la inducción de NGF por otros inductores como la vitamina D3 (Rush et al., 1995). En el exón 3 existe además un segundo promotor capaz de inducir la transcripción (Racke et al., 1996).

El mRNA del NGF posee en el extremo 3' una secuencia no codificante rica en AU, denominada 3'UTR, que actúa en cis determinando la elevada inestabilidad del mRNA de NGF, posiblemente a través de la interacción en trans con proteínas de unión a RNA (Tang et al., 1997). La inestabilidad del mRNA de NGF condiciona una vida media de los transcritos muy corta (Onteniente et al., 1994), lo que explicaría el hecho de que numerosos agentes inductores de la síntesis del NGF, ejerzan su acción a través de la activación de la transcripción del gen de NGF (Carswell, 1993; Racke et al., 1996).

2.2 LA PROTEÍNA NGF

El NGF se sintetiza como un precursor, preproNGF, de 34 kDa a partir del transcrito A. Tras la hidrólisis de los 25 aminoácidos del péptido señal, se genera el proNGF, y a partir de este el β-NGF maduro, responsable de la actividad biológica. La forma madura del β-NGF está formada por dos cadenas idénticas asociadas de forma no covalente y con tres puentes disulfuro intracatenarios. El β-NGF se encuentra formando parte de un complejo multiprotéico de dos subunidades α, un homodímero β, y dos subunidades γ. La subunidad γ es una arginina peptidasa responsable del procesamiento del β-NGF (Edwards et al., 1988). La función exacta de la subunidad α se desconoce pero se cree que protege el β-NGF de la degradación enzimática. Todos los determinantes necesarios para el adecuado proceso de maduración y secreción del NGF se conservan en la secuencia del NGF maduro (Heymach et al., 1996). El NGF sintetizado es inmediatamente secretado, siendo los niveles intracelulares de NGF muy pequeños (Furukawa et al., 1987). En células de naturaleza neuroendocrina, la secreción puede ocurrir por gránulos de secreción y estar bajo control de neurotransmisores (Missale et al., 1996). Se han descrito recientemente anticuerpos naturales antiNGF que podrían tener una función transportadora del NGF en suero (Dicou y Nerriere, 1997).

Estudios cristalográficos han permitido determinar la estructura tridimensional del NGF, que incluye dos láminas β antiparalelas unidas por tres cortos segmentos de gran variabilidad conformacional (McDonald y Chao, 1995). Los tres puentes disulfuro se agrupan en un motivo estructural denominado nudo de cisteínas. Estu-

dios de funcionalidad por mutagénesis dirigida han permitido identificar los aminoácidos necesarios para la unión específica y activación del receptor (Woo y Neet, 1996).

2.3 LOCALIZACIÓN DE LA SÍNTESIS DEL NGF

Tejidos periféricos

La síntesis de NGF en los tejidos periféricos se correlaciona de forma positiva con el grado de inervación simpática o sensorial (Korsching y Thoenen, 1983; Davies et al., 1987). Tejidos como la piel, el iris, o el corazón tienen un contenido relativamente alto de NGF y NGF mRNA (Heumann y Thoenen, 1986). La síntesis de NGF es también significativa en próstata, testículos, tiroides y tejido adiposo pardo (Nisoli et al., 1996; Chen et al., 1997).

El NGF sintetizado en los tejidos diana alcanza los ganglios simpáticos y sensoriales por transporte retrógrado (Korsching y Thoenen, 1988; von Bartheld et al., 1996). En estos ganglios, al igual que en el nervio ciático, los niveles de mRNA son muy bajos, pero el contenido en NGF es elevado. La axotomía del nervio ciático produce un aumento bifásico en los niveles de NGF mRNA (Heuman et al., 1987), primero por la liberación de factores séricos (Spranger et al., 1990; Neveu et al., 1992), y posteriormente por la secreción de citoquinas, fundamentalmente IL-1β por la invasión del nervio por macrófagos activados (Hattori et al., 1993; Pshenichkin y Wise, 1995; Dekosky et al., 1996).

Sistema nervioso central

De manera similar a lo que ocurre en el sistema nervioso periférico (SNP), el NGF es sintetizado por los tejidos diana de las neuronas colinérgicas del cerebro basal anterior, que constituyen las principales células sensibles al NGF (Robner et al., 1997). Así, la síntesis está directamente relacionada con el grado de inervación colinérgica (Korsching et al., 1985; Robner et al., 1997). Mientras que en el SNP la síntesis se realiza por células no neuronales, en SNC la síntesis tiene lugar en las neuronas, encontrándose elevados niveles de NGF en el hipocampo y en menor medida en la corteza y bulbo olfativo (Korsching et al., 1985; Ayer-Lelievre et al., 1988; Rocamora et al., 1996). Las neuronas constituyen la fuente mayoritaria de NGF en el SNC adulto y ejercen un efecto represor de su expresión en astrocitos (Vigé et al., 1992), pero durante el desarrollo o en caso de lesión, los astrocitos pueden expresar el NGF de forma significativa (Chakrabarti et al., 1990; Lu et al., 1991; Arendt et al., 1995).

Síntesis de NGF "in vitro"

La síntesis de NGF *in vitro* se produce en una gran diversidad de tipos celulares. Las neuronas procedentes de distintas localizaciones del SNC como hipocampo, septo, estriado y corteza sintetizan NGF pero con diferente intensidad (Houlgatte et al., 1989). Los cultivos primarios de células gliales son también capaces de sintetizar NGF (Neveu et al., 1992; Jehan et al., 1995; Brodie, 1996; Boutros et al., 1997), sin que la procedencia de los astrocitos condicione los niveles de síntesis (Houlgatte et al., 1989). Cultivos primarios de células cardiácas, células musculares lisas, células de Schwann, células mesangiales (Plüss et al., 1995; Creedon y Tuttle, 1997) además de macrofagos y linfocitos T CD 4^+, entre otras células del sistema inmune, son capaces de producir NGF (Lambiase et al., 1997). Para el estudio de la regulación del gen de NGF se han utilizado líneas derivadas de fibroblastos, L929 o 3T3 (Wion et al., 1990; D'Mello y Heinrich, 1991b). El NGF es producido además, por células de glioma C6 (Fukumoto et al., 1994; Colangelo et al., 1996), células neuroendocrinas, neuroblastos y líneas osteoblásticas (Charrasse et al., 1992; Jehan et al., 1996; Heymach et al., 1996).

2.4 REGULACIÓN DE LA SÍNTESIS DE NGF

La regulación de la síntesis de NGF es el principal mecanismo de regulación de los niveles de NGF extracelulares (Carswell et al., 1993; Racke et al., 1996) y por ello su conocimiento resulta de suma importancia teniendo en cuenta el potencial terapéutico de las neurotrofinas en general y del NGF en particular (Carswell, 1993; Rush et al., 1995). Dado que el NGF no es capaz de atravesar la barrera hematoencefálica, no es posible su inyección sistémica para tratar de paliar determinadas situaciones neuropatológicas (Han et al., 1997). La investigación aplicada en esta área va encaminada a identificar posibles moduladores de la expresión del NGF o de otras neurotrofinas y la utilización de células genéticamente modificadas que podrían ser encapsuladas y transplantadas.

La síntesis de NGF por células en cultivo depende del estado de crecimiento celular. Así, en cultivos no confluentes, los niveles de síntesis de NGF son elevados, reduciéndose la síntesis conforme se alcanza la confluencia debido a una inhibición por contacto celular (Furukawa et al 1987; Houlgatte et al., 1989; Lu et al., 1991). En el proceso de gliosis, que sigue a un proceso de lesión, se liberan factores mitogénicos y citoquinas pro-inflamatorias que explican una inducción de la síntesis y secreción

de NGF tanto *in vivo* como *in vitro* (Arendt et al., 1995; Psenichkin y Wise, 1995; Dekosky et al., 1996).

Factores proliferativos

La utilización de medios de cultivo celular químicamente definidos permitió poner en evidencia el efecto estimulador del suero en la síntesis de NGF. El efecto del suero es parcialmente dependiente de proteínas G sensibles a toxina pertusis y ha sido observado tanto en fibroblastos como en astrocitos (Spranger et al., 1990; Neveu et al., 1992). Como en SNC los astrocitos no están en contacto con el plasma, el efecto inductor del suero podría reflejar la situación tras una lesión vascular, que puede ser imitada *in vitro* por el modelo experimental de lesión del nervio ciático (Heumann et al., 1987). La expresión de c-fos precede al aumento de la síntesis de NGF por suero y por lesión del nervio ciático (Hengerer et al., 1990). La inducción de la síntesis de NGF por el suero es dependiente de la activación de cPKCs. En este sentido el PMA es también un potente inductor de la síntesis de NGF, y la regulación por los dos inductores tiene características comunes (Neveu et al., 1992). I*n vivo,* parece haber un equilibrio entre la regulación positiva que ejerce la PKC en la expresión del NGF y el efecto inhibidor que ejercen los glucocorticoides (Neveu et al., 1992). La inducción de NGF dependiente de PKC se inhibe por un aumento en la concentración intracelular de Ca^{2+}, lo que se puede explicar por los distintos requerimientos de Ca^{2+} de las diferentes isoformas de PKC, o por efecto del Ca^{2+} en la "downregulation" de cPKCs (Jehan et al., 1995). Los factores de crecimiento fueron algunos de los primeros estimuladores conocidos de la síntesis del NGF y el EGF, FGFa/b y el TGFβ estimulan la producción de NGF por astrocitos (Yoshida y Gage, 1992).

Factores proliferativos de astrocitos como la trombina y los nucleótidos de guanina, también estimulan la síntesis y secreción de NGF (Middlemiss et al., 1995; Debeir et al., 1996). La inducción por trombina es dependiente de PKC y fisiológicamente puede estar relacionada con la exposición de las células gliales a proteasas tras un proceso traumático.

Mediadores de la inflamación

La IL-1β y el TNFα son importantes citoquinas reguladoras de la respuesta inflamatoria. A nivel periférico son sintetizadas por los macrófagos activados, y en el SNC por la microglía y por los astrocitos reactivos (Merril y Benveniste, 1996). Estas dos citoquinas pro-inflamatorias son potentes estimuladores de la síntesis y secreción

de NGF. Así, la IL-1β es la responsable de la inducción de NGF que ocurre *in vivo* tras la lesión del nervio ciático o un trauma cortical (Heumann et al., 1987; Dekosky et al., 1996). Además, la inducción de NGF por IL-1β y TNFα se observa también *in vitro* en astrocitos (Carman-Krzan y Wise, 1993; Psenichkin y Wise, 1995). La inducción de NGF no está restringida a células gliales, habiéndose observado también en fibroblastos y cultivos primarios de hipocampo formados por neuronas y células gliales (Hattori et al., 1993;1996).

La regulación de la producción de NGF ejercida por la IL-1β ocurre a diferentes niveles: aumento de la transcripción y estabilización de los transcritos formados. El efecto de la IL-1β es independiente de la actividad de cPKCs (Psenichkin et al., 1994) y no se ve afectado por el aumento en los niveles intracelulares de cAMP, (Carman-Krzan y Wise, 1993; Han et al., 1994), ni tampoco por el aumento en la concentración intracelular de Ca^{2+} (Friedman et al., 1992). Parece que en general, la hidrólisis de fosfoinosítidos tiene poca importancia en la regulación de la síntesis del NGF (Jehan et al., 1995), y el efecto de la IL-1β es también independiente de esta ruta de señalización celular (Friedman et al., 1992).

El efecto de la IL-1β se atribuye a la generación de ácido araquidónico vía activación de PLA_2, que posteriormente es metabolizado a leucotrienos (Carman-Krzan y Wise, 1993). En lo que se refiere al TNFα, la inducción de NGF parece estar mediada por el receptor de 55 kDa, que es el receptor mayoritario en astrocitos (Hattori et al., 1996; Dopp et al., 1997).

Las catecolaminas

La activación de receptores β-adrenérgicos, o el aumento de los niveles intracelulares de cAMP, puede ejercer en determinadas circunstancias un efecto activador de la síntesis y secreción de NGF tanto *in vivo* como en células gliales (Condorelli et al., 1994; Fukumoto et al., 1994). Sin embargo, en otras circunstancias carece de efecto (Friedman et al., 1992; Neveu et al., 1992), o ejerce un efecto supresor de la inducción por suero, PMA o TGF-β1 (Han et al., 1994; Jehan et al., 1995). Las diferencias en la respuesta a agonistas adrenérgicos parecen ser debidas a diferencias en el estado de crecimiento del cultivo celular (Furukawa et al., 1987; Han et al., 1994).

Regulación de la síntesis de NGF por diversos factores

La síntesis y secreción de NGF *in vitro* se puede modular por multitud de factores, a continuación se citan únicamente algunos de ellos. Citoquinas como IL-4, IL-

5, IL-10 y el factor activador de plaquetas poseen un efecto estimulador de la síntesis de NGF (Awatsuji et al., 1993; Brodie, 1996), sin embargo el interferón γ ejerce un efecto inhibidor de la síntesis de NGF (Awatsuji et al., 1995). El interferón β y $PDGF_{BB}$ pueden estimular o inhibir la producción de NGF en función de las condiciones y tipo de cultivo celular utilizado (Plüss et al., 1995; Boutros et al., 1997; Creedon y Tuttle, 1997).

La 1,25-dihidroxivitamina D3 ejerce un efecto estimulador de la síntesis de NGF en distintos modelos celulares, entre ellos astrocitos y células de glioma C6 (Neveu et al., 1994; Jehan et al., 1996; Han et al., 1997). La vitamina D3 posee un efecto aditivo en la inducción de NGF por IL-1β, sinérgico con el TGF-β1 y contrarresta el efecto supresor que ejerce la corticosterona (Pshenichkin et al., 1994; Han et al., 1997). La activación de la microglía en un proceso traumático del SNC podría explicar un aumento local en los niveles de vitamina D3 que estimularía la síntesis de NGF por los astrocitos (Neveu et al., 1994). Los glucocorticoides regulan la síntesis de NGF de modo complejo y específico del tipo celular. En astrocitos, los glucocorticoides inhiben la secreción basal e inducida por PMA, suero, vitamina D3, o citoquinas proinflamatorias (Neveu et al., 1992; Psenichkin et al., 1994; Han et al., 1997).

La expresión del gen de NGF está controlada además por el neurotransmisor glutamato a través de la inducción de NO, la organización del citoesqueleto y el estrés oxidativo (Naveilhan et al., 1994; Persichini et al., 1994; Baudet et al., 1995).

3. LOS ASTROCITOS Y OTRAS CELULAS GLIALES

Los astrocitos son las células no neuronales mayoritarias del SNC, y se agrupan junto con los oligodendrocitos y la microglía en el término general de células gliales (revisado por Porter y McCarthy, 1997). Existen dos tipos morfologicamente diferentes de astrocitos, protoplásmicos situados en la materia gris y astrocitos fibrosos fundamentalmente en las fibras mielínicas.

En 1983 Raff y colaboradores demostraron la existencia de dos tipos de astrocitos en cultivos de nervio óptico perinatal procedentes de dos tipos celulares diferentes. Estos estudios pusieron de manifiesto la existencia de un progenitor celular O-2A que puede diferenciarse en oligodendrocitos y astrocitos de tipo 2. Las células progenitoras O-2A se caracterizan por ser reconocidas por un anticuerpo monoclonal A2B5, y se diferencian en presencia de suero mayoritariamente en astrocitos de tipo 2, mientras en un medio químicamente definido se diferencian en oligodendrocitos (Temple y Raff, 1985). La proteína glial fibrilar ácida (GFAP), constituyente de los

filamentos intermedios, es un marcador específico de los astrocitos. Los astrocitos tipo 1 en cultivo son células de aspecto poligonal, no radial y tienen un origen diferente de los de tipo 2. Los astrocitos de tipo 1 se caracterizan por expresar GFAP y la glicoproteína Ran-2 ($GFAP^+/Ran\text{-}2^+/A2B5^-$)(Wilkin et al., 1990). Los astrocitos de tipo 2, originarios de células progenitoras O-2A, son $GFAP^+/Ran\text{-}2^-/A2B5^+$ y poseen una morfología estrellada.

Los astrocitos constituyen un tipo celular especialmente heterogéneo, existiendo importantes diferencias funcionales entre astrocitos tipo 1 y 2, además de diferencias entre células de diferente origen anatómico (Mangoura et al., 1995). La heterogeneidad astrocítica es reflejo de la heterogeneidad neuronal e igualmente esta se ve influenciada por la diferente funcionalidad astrocitaria (Kimelberg, 1995).

Los astrocitos realizan un gran número de funciones que permiten un adecuado funcionamiento del SNC. En el desarrollo, durante la formación del tubo neural, los astrocitos dirigen la migración de las neuronas, producen factores neurotróficos y de crecimiento fundamentales en el desarrollo y supervivencia neuronal. Los astrocitos configuran el espacio sináptico, y mantienen una comunicación bidireccional con las neuronas a través de los neurotransmisores (Kimelberg, 1995; Porter y McCarthy, 1997). Los astrocitos regulan la homeostasis del medio extracelular, modificando las concentraciones iónicas, fundamentalmente de K^+, regulando el pH y participando en la recaptura de determinados neurotransmisores (glutamato, catecolaminas). Además, proporcionan substratos metabólicos esenciales para el metabolismo neuronal (Magistretti y Pellerin, 1996; Giaume et al., 1997). Los astrocitos son células que responden a los procesos de traumatismo cerebral, en caso de lesión se activan en un proceso gliótico y en este estado "reactivo" sobreexpresan neurotrofinas, citoquinas, factores de crecimiento y moléculas de adhesión (Arendt et al., 1995; Hurwitz et al.

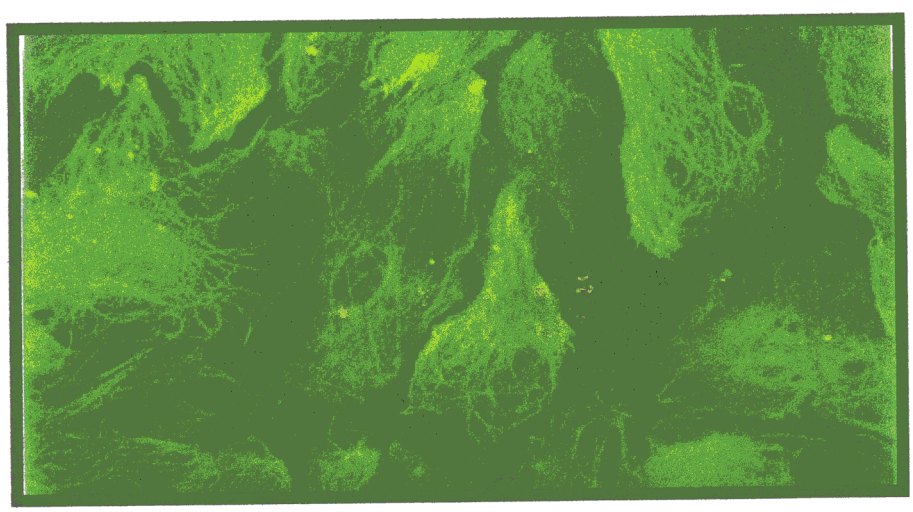

Fig. 6 Marcaje por inmunofluorescencia de GFAP en cultivo primario de astrocitos

1995). De este modo en situaciones de traumatismo, lesión, infección bacteriana o viral los astrocitos pueden proteger, favorecer la regeneración del tejido dañado o regular el proceso inflamatorio. Los astrocitos reactivos pueden expresar el complejo mayor de histocompatibilidad y actuar como células presentadoras de antígeno en la respuesta inmune mediada por linfocitos T (Hurwitz et al., 1995; Nikcevich et al., 1997).

Las células de glioma C6

Las células C6 de glioma proceden de un glioma de rata inducido químicamente y se caracterizan por su naturaleza indiferenciada con potencial astrocítico y oligodendrocitario. Las células C6 pueden expresar GFAP y se utilizan como modelo en el estudio de la regulación de la síntesis de NGF (Fukumoto et al., 1994; Colangelo et al., 1996). Además se utilizan como modelo celular en el estudio de los mecanismos de transducción de señales en células gliales (Chen et al., 1995; Anciaux et al., 1997; Yoshimura et al., 1997a;b).

4. OBJETIVOS

Del NGF, la primera neurotrofina descubierta, existe un conocimiento pormenorizado sobre su funcionalidad y estructura. El papel del NGF en el normal funcionamiento del sistema nervioso, y su posible utilidad terapéutica han impulsado el estudio de los mecanismos de síntesis de este factor. Esta área de investigación ha permitido identificar agentes moduladores de la síntesis de NGF en distintos modelos experimentales. La presente tesis doctoral pretende profundizar en el estudio de los mecanismos de señalización celular conducentes a la activación de la síntesis y secreción de este factor neurotrófico en células gliales, estableciéndose los siguientes objetivos:

1. El estudio de mecanismos de transducción de señales implicados en la regulación de la síntesis de NGF, centrado en la generación de mensajeros lipídicos intracelulares clásicos y en la recientemente caracterizada ruta de las ceramidas.

2. Caracterización de los posibles sistemas de fosforilación con los que está coordinada la generación de mensajeros lipídicos y de los factores de transcripción implicados.

3. Establecimiento del posible papel de cada uno de los niveles anteriormente mencionados en el mecanismo de acción de estimuladores de la expresión de NGF.

II- RESULTADOS Y DISCUSIÓN

1. REGULACIÓN DE LA SÍNTESIS Y SECRECIÓN DE NGF POR MENSAJEROS LIPÍDICOS EN CULTIVOS PRIMARIOS DE ASTROCITOS

Con este capítulo se introduce el tema principal objeto de esta tesis doctoral, el papel de mensajeros de naturaleza lipídica en la regulación de la síntesis de NGF por células gliales. El estudio de los mensajeros intracelulares de origen lipídico y su mecanismo de acción ha sido una de las áreas de la bioquímica que más ha avanzado durante los últimos años, proporcionando nuevos conocimientos sobre el funcionamiento celular (Prescott, 1997). Así, se ha pasado de considerar a los lípidos de membrana como componentes poco más que estructurales a importantes reguladores de las funciones celulares. El trabajo se centra primeramente en el estudio del efecto de la adición exógena de PC-PLC sobre la síntesis y secreción de NGF en cultivos primarios de células gliales. La acción estimuladora de la PC-PLC en la síntesis de NGF sugiere un posible papel de la ruta de la SMasa-ceramida, puesto que ambos sistemas han sido directamente relacionados (Schütze et al., 1992; Müller et al., 1994; Wiegman et al., 1994). Se estudia a continuación la importancia de la ruta de señalización por ceramidas en la regulación de la síntesis de NGF, así como los posibles sistemas de transducción de señales implicados. La utilización de análogos permeables de ceramida, o la adición de SMasa exógena desencadena una cascada de procesos intracelulares que conducen a un aumento en la producción de NGF. Los resultados que se exponen evidencian la existencia de una regulación cruzada de la síntesis de NGF entre vías de señalización que involucran tanto a glicerolípidos como a esfingolípidos.

Phosphatidylcholine-phospholipase C mediates the induction of nerve growth factor in cultured glial cells

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Abstract **Addition of phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) to cultured glial cells increased the levels of nerve growth factor (NGF) mRNA and the amount of cell-secreted NGF. The effect of PC-PLC was 2.5 times higher than that elicited by 4β-phorbol 12β-myristate 13α-acetate. In cells in which protein kinase C (PKC) was fully inhibited or down-regulated, the effect of PC-PLC was reduced – though still evident – and similar to that exerted by sphingosine. Results thus indicate that PC-PLC induces the synthesis of NGF by glial cells by a PKC-dependent and PKC-independent mechanisms.**

Key words: Nerve growth factor; phosphatidylcholine; Protein kinase C; Sphingosine; Glial cell; Astrocyte

1. Introduction

Nerve growth factor (NGF) is a neurotrophic protein required for the development and survival of several populations of neurons [1]. In the central nervous system, NGF is synthesized by certain neurons, but during brain development and under different pathological alterations of the nervous system, there is a local increase in the production of NGF by astrocytes [2–5]. Thus, substantial present effort is directed toward an understanding of the influences that control NGF synthesis, because of the possibility that increasing the endogenous synthesis of NGF might provide clinical advantage in degenerative diseases of central nervous system [6]. However, little is known about the effectors that induce the synthesis of NGF by those cells and about how this synthetic process is up-regulated. It seems that several NGF-inducing agents act through different pathways. One of them is the signalling network involving the activation of protein kinase C (PKC). Studies performed with cultured astrocytes and fibroblasts have shown that 4β-phorbol 12β-myristate 13α-acetate (PMA), a well-documented PKC activator, induces the synthesis and secretion of NGF, and this effect is counteracted by PKC inhibitors like H-7 or H-9 [7,9]. PMA induces the expression of c-*fos*, the product of which enhances NGF synthesis by the same route as PMA [5,9,10].

PKC is physiologically activated by diacylglycerol (DAG) resulting from agonist-induced hydrolysis of inositol phospholipids [11]. However, this DAG rapidly disappears and seems to be responsible for a transient activation of PKC [12]. In contrast, hydrolysis of other membrane phospholipids, particularly phosphatidylcholine (PC), produces DAG at a relatively later phase. This DAG may be the responsible for the sustained activation of PKC that leads to long-term cellular responses [13]. Mechanisms of cellular response to PC hydrolysis have been demonstrated to occur in many cell types (reviewed in [12,14]). Despite extensive studies, the biochemical mechanisms underlying signal-induced activation of PC breakdown are still poorly defined. Phospholipase C (PLC)-catalyzed hydrolysis of PC has been shown to be activated in response to a number of agonists [15], in transformed cells [16] and in signalling cascades triggered by growth factors [17]. PC-PLC provides a positive signal for PKC by inducing its translocation to membrane though not its down-regulation [16]. It has also been shown that PC hydrolysis by a PC-PLC mimics the ability of carbachol to inhibit adenylyl cyclase [18]. Furthermore, a role of PC-specific phospholipase D (PLD) in signal transduction has been described [13,19].

In the present study we investigated the possible relationship between PC hydrolysis and PKC activation in the control of NGF synthesis by astrocytes. Previous experimental evidence showed that activation of PKC with PMA stimulated NGF synthesis by glial cells [7] while exogenously added PC-PLC was able to activate PKC in fibroblasts [18]. This provinded us a tool to investigate whether NGF synthesis by glial cells depends on PC breakdown.

2. Experimental

2.1. Culture conditions

Primary cultures of rat brain glial cells were prepared from cerebral hemispheres of 1–2-day-old rat pups. Cerebral hemispheres were dissected in phosphate buffered saline (PBS) supplemented with 0.33% glucose and treated with trypsin (5 mg/ml, 30 min at 37°C). Trypsin was subsequently inhibited by the addition of 10% foetal calf serum (FCS), before treatment with DNase I (10 μg/ml, 5 min at 37°C). Brain cells were dissociated by fluxation with a Pasteur pipette and next sedimented by low speed centrifugation (1000 × *g*, 5 min). The pellet was gently resuspended, and cells were seeded at a density of 3×10^4 cells/cm^2. All plastic supports were previously coated with 5 μg/ml poly-L-ornithine in water. Cells were cultured for 3 weeks in basal medium containing a mixture of Dulbecco's modified Eagle medium and Ham's F12 (1/1, v/v), supplemented with 0.66% glucose, 5 μg/ml streptomycin, 5 U/ml penicillin and 10% FCS. Three days before the experiment, FCS was removed and cells were transferred to a chemically defined medium consisting of basal medium supplemented with 25 μg/ml insulin, 100 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine and 30 nM sodium selenite. Immunocytochemical studies showed that 90% of cultured cells were positive for glial fibrillary acidic protein, indicating that cultures were largely enriched in astrocytes.

2.2. Assay of NGF and NGF mRNA

Cell supernatants were collected 24 h after the different treatments described in the text and diluted in 1 volume of PBS containing 0.1% Tween-20 and 0.5% gelatin. NGF released by the cells was assayed in triplicate, with a double-site-ELISA, using a monoclonal anti-NGF antibody, coupled or not to β-galactosidase (Boehringer Manheim), according to [20]. Northern blot analysis was performed by standard procedures [7]. After 6 h of treatment, total RNA was extracted from

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cells by the LiCl/urea method. Glyoxal-treated RNAs were fractionated in agarose gel, transferred to a nylon membrane by capillary blotting, and hybridized with a ^{32}P-labelled NGF cDNA and a amyloid precursor protein (APP) cDNA, used to verify the loading of the gels.

2.3. Identification of PKC by immunoblotting

Glial cells (treated as described in the legend to Fig. 3) were scraped into 0.5 ml of ice-cold 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM 2-mercaptoethanol containing 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml benzamidin, 5 μg/ml soybean tripsin inhibitor and 0.1 mM PMSF, and rapidly centrifuged at 5,000 × *g* for 5 min to eliminate debries. Following denaturation in SDS sample buffer, proteins were resolved in a 8% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% of fat-free dried milk and incubated with 2 μg/ml of specific anti-PKC antiserum (clone MC5, Amersham International). Following incubation with horseradish conjugated anti-mouse antibody, the blots were developed with an enhanced chemiluminescence detection kit (Amersham International).

2.4. Other chemicals

Tissue culture plastic wares were purchased from Nunc (Denmark), culture media from Gibco (France) and FCS from Eurobio (France). PC-PLC and bisindolylmaleimide (GF 109203X) were from Calbiochem (USA). Sphingosine and most other reagents were obtained from Sigma Chemicals (USA).

3. Results

3.1. PC-PLC enhances the synthesis of NGF

Cultured glial cells were treated for 24 h with different doses of exogenously added PC-PLC from *Bacillus cereus* and NGF levels were determined in the supernatant media 24 h later. Results presented in Fig. 1 show a dose-dependent and saturable increase in the extracellular levels of NGF, which become maximal at 0.5 U/ml of PC-PLC. Doses over 1 U/ml were toxic to cells. Stimulation of cells with 100 nM of PMA was quantitatively lower than that induced by PC-PLC (Fig. 1).

In order to determine whether PC-PLC had a corresponding on the pool of NGF transcripts, total RNA was extracted from cells pretreated with or without the enzyme. Northern blot analysis showed that PC-PLC produced a marked increase in

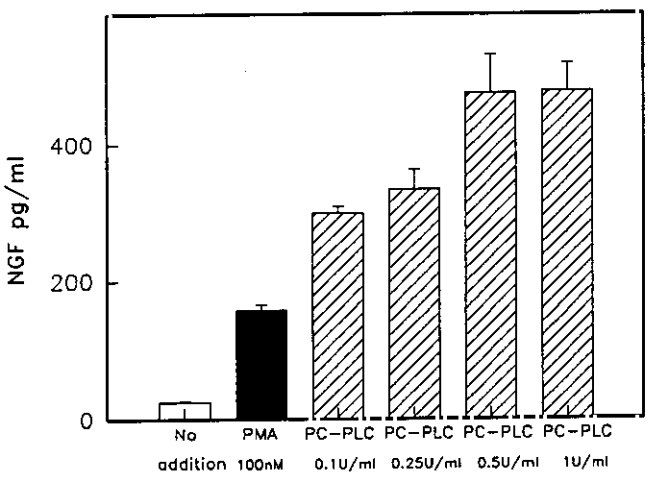

Fig. 1. Effect of increasing concentrations of PC-PLC on the production of NGF. Glial cells were incubated with no additions, PMA (100 nM), or PC-PLC (0.1 U/ml, 0.25 U/ml, 0.5 U/ml and 1 U/ml). The medium was collected after 24 h and NGF was quantified by double-site ELISA. Values are means ± S.D. of 3 independent experiments assayed in triplicate.

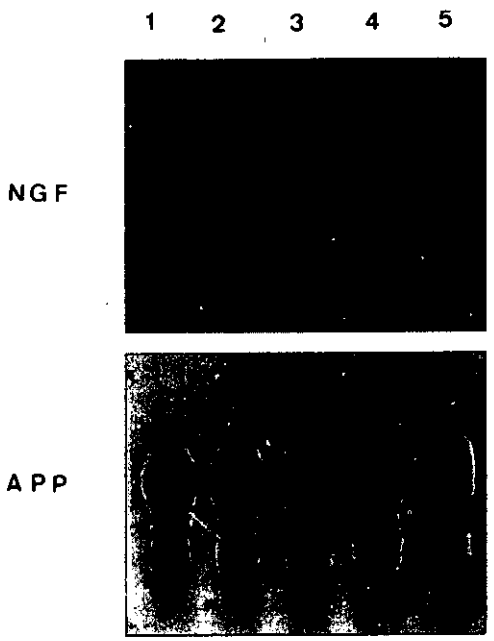

Fig. 2. Accumulation of NGF mRNA in astrocytes. Constant amounts of total RNAs were fractionated on an agarose gel, blotted and hybridized with a radiolabelled NGF cDNA probe. Lane 1: control; lane 2: PMA 100 nM; lane 3: PC-PLC 0.5 U/ml; lane 4: cells treated with PMA 500 nM for 48 h and the last 24 h with PMA 100 nM; and lane 5: cells treated with PMA 500 nM for 48 h and the last 24 h with PC-PLC 0.5 U/ml. Essentially identical results were obtained in two independent experiments.

NGF mRNA, as compared to controls, indicating that PC-PLC is able to promote NGF synthesis (Fig. 2, lanes 1 and 3). This effect is more potent than that elicited by PMA (Fig. 2, lane 2).

3.2. The induction of NGF synthesis by PC-PLC is partially dependent on PKC

To investigate whether PKC activation is required for the PC-PLC-triggered induction of NGF synthesis, PKC was down-regulated in glial cells by pretreating the cell cultures with a supramaximal dose of PMA (500 nM) for 48 h. This treatment completely removed PKC from astrocytes as determined by Western blot analysis with a specific monoclonal anti-PKC antibody (Fig. 3). Interestingly, induction of NGF synthesis by PC-PLC emerged reduced but not abolished, in cells in which PKC was down-regulated (Table 1), whereas NGF induction

Table 1
Effect of PKC inhibition and down-regulation and effect of sphingosine on the levels of cell-secreted NGF measured by ELISA

Additions	NGF pg/ml
None	34.68 ± 10
PMA 100 nM	196.51 ± 90
PC-PLC 0.5 U/ml	490.54 ± 119
PMA 100 nM + BIM 2 μM	37.41 ± 3
PC-PLC 0.5 U/ml + BIM 2 μM	130.30 ± 23
PMA 500 nM (48 h) + PMA 100 nM	32.87 ± 2
PMA 500 nM (48 h) + PC-PLC 0.5 U/ml	171.50 ± 27
Sphingosine 10 μM	164.80 ± 12

The values represent the means ± S.E.M. of 2 independent experiments assayed in triplicate.

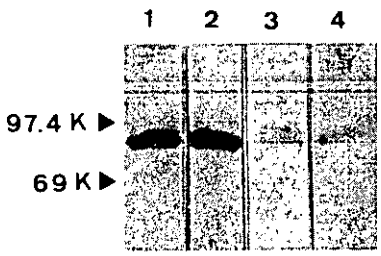

Fig. 3. Western blot analysis of PKC levels in cell lysates. Lane 1: control; lane 2: PMA 100 nM; lane 3: cells treated with PMA 500 nM for 48 h and the last 24 h with PMA 100 nM; and lane 4: cells treated with PMA 500 nM for 48 h and the last 24 h with PC-PLC 0.5 U/ml.

as elicited by PMA was completely abolished in down-regulated cells. This effect was also observed at the mRNA levels, as determined by Northern blot analysis (Fig. 2, lanes 4 and 5).

Further experimental evidence to support these data was obtained by the use of bisindolylmaleimide (BIM), a potent and higly specific inhibitor of PKC [21]. Inhibition of PKC by BIM completely abolished the increase in NGF levels induced by PMA, whereas treatment of cells with BIM only reduced in part the increase in NGF levels produced by PC-PLC (Table 1). Hence, PKC seems to be only partially involved in the NGF inducing response elicited by PC-PLC.

3.3. *Sphingosine increases the production of NGF*

In order to study whether another mechanism of signal transduction could be involved in the synthesis of NGF, we determined the effect of sphyngosine on NGF secretion by glial cells. Results reported in Table 1 show that sphingosine increases the extracellular levels of NGF to the same extent as PC-PLC does in PKC down-regulated cells.

4. Discussion

Despite the recent efforts performed to unravel the mechanisms of NGF induction, the signal-transducing pathways leading to the synthesis of this neurotrophic protein remain largely unknown. Many of the agents which are able to stimulate NGF synthesis are dependent on PKC activation [9]. However, steroids and 1,25-dihydroxyvitamin D_3 have been shown to induce NGF protein and mRNA by a mechanism different to serum and PMA, and independent of PKC and c-*fos* gene activation [22,24]. Although this indicates that the the control of NGF synthesis does not depend on a unique pathway, the different putative mechanisms up-regulating NGF synthesis operate by increasing the steady-state levels of intracellular NGF mRNA.

In this report we demonstrate that a PC-PLC induces the synthesis of NGF by glial cells by a mechanism that is partially dependent on PKC activation. The fact that PC-PLC is able to produce an accumulation of NGF transcripts indicates that at least part of the stimulatory effect takes place at the pre-translational level. One of the particular features of the action of PC-PLC, alone or in combination with phosphatidate phosphohydrolase, is that the increase in intracellular DAG concentration is prolonged, indicating that it may be responsible for long-term cellular responses [13]. Our results are in agreement with this notion since NGF is generally considered to be coded by a late expression gene. Results also indicate that PC-PLC acts by at least two different pathways, one dependent on PKC and another independent of PKC. We and others have previously found that PC-PLC triggers some cellular responses, namely induction of DNA synthesis or activation of nuclear transcription factor systems by a PKC-independent mechanism [17,24]. It has been described that PC-PLC is coupled to a sphingomyelinase that catalyzes the breakdown of sphingomyelin into ceramide [24]. A second messenger function for the sphingomyelin cycle and ceramide in the control of cell growth, differentiation and apoptosis has been recently proposed [25]. Our data show that sphingosine induces NGF synthesis to a similar extent than the PKC-independent mechanism elicited by PC-PLC. The effect exerted by sphyngosine may be mediated by its conversion to ceramide, as it has been suggested for other biological responses mediated by sphingosine [26]. On the other hand, vitamin D_3, which induces NGF by a PKC-independent mechanism, has been shown to elicit an early and reversible hydrolisis of sphingomyelin with a concomitant generation of ceramide [27]. Two mechanisms may be therefore involved in the generation of NGF by astrocytes, one represented by serum and DAG acting through PKC, and another one induced by vitamin D_3 acting through the sphingomyelin cycle. Our results suggest that the breakdown of PC by a PLC may trigger both mechanisms.

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Research report

Induction of nerve growth factor synthesis by sphingomyelinase and ceramide in primary astrocyte cultures

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Astrocytes synthesize nerve growth factor (NGF) in response to pro-inflammatory cytokines. To further study the signaling mechanism involved in this induction of NGF production, the sphingomyelin (SM) pathway was studied. Addition of exogenous neutral SMase (*Staphylococcus aureus*) or C_2-ceramide to primary cultures of newborn rat cortical astrocytes elicited a dose-response increase of NGF synthesis, with maximal effect at 1 U/ml and 25 μM, respectively. Induction of NGF synthesis by SMase and ceramide was shown to be independent of classical PKC activity. Intracellular cAMP-raising agents, such as forskolin and 3-isobutyl-1-methylxanthine, partially prevented the SMase- and C_2-ceramide-induced secretion of NGF to the cell supernatant. PD098059 and apigenin, inhibitors of the mitogen-activated protein (MAP) kinase pathway, produced a dose-response inhibition of the SMase- and C_2-cer-induced release of NGF. This observation points to the possibility that regulation of NGF synthesis and secretion by the SMase pathway may be mediated downstream by the MAP kinase cascade. As a matter of fact, pretreatment of astrocytes with SMase or C_8-ceramide led to an increased phosphorylation of raf-1. Moreover, MAP kinase activity was enhanced in astrocytes treated with SMase or both ceramides In conclusion, results suggest that the SMase pathway may control NGF synthesis in the central nervous system, and raise the possibility of an involvement of the MAP kinase cascade in this process.

Key words: Astrocyte, Ceramide, Mitogen-activated protein kinase, Nerve growth factor, Raf-1, Sphingomyelinase.
Abbreviations: BIM, bisindolylmaleimide; cAMP, cyclic AMP; C_2-cer, N-acetylsphingosine; FK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; IL, interleukin; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; PMA, 4β-phorbol 12β-myristate; SM, sphingomyelin; TNF, tumor necrosis factor.

1. Introduction

Within the last decade sphingolipids have emerged as active participants in the regulation of a wide range of cellular responses. In particular, sphingomyelin (SM) breakdown has been implicated in the regulation of cell growth, differentiation, transformation and apoptosis. SM is hydrolyzed by sphingomyelinase (SMase), releasing diffusible ceramides that function as second messengers in signaling pathways [reviewed in 12, 13, 33, 34]. Ceramides may modulate the activity of different serine/threonine protein kinases as ceramide-activated protein kinase (CAPK) and protein kinase C ζ, as well as ceramide-activated protein phosphatase (CAPP) [13]. Downstream signaling occurs at least in part through the mitogen-activated protein (MAP) kinase cascade and NFκB translocation [2, 13, 29, 33, 38]. An increasing number of cell-surface receptors are currently being show to generate signals that trigger SM breakdown. Accumulation of ceramides derived from SM hydrolysis has been described to occur in response to different extracellular agents including 1α,25-dihydroxyvitamin D_3, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-2, and interferon-γ [13, 33, 34].

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TNFα and ILs are pivotal mediators of inflammation and immune responses in the central nervous system (CNS). Such responses are followed by the rapid activation of brain resident macrophages and subsequent activation of astrocytes, that constitute the most abundant non-neural cells in the CNS [23]. Astrocytes play an important role in a wide range of functions which are essential for adequate development, functionality, and pathological process of the CNS [3]. It is now well established that astrocytes are involved in the inflammatory response in the brain like that occuring in multiple sclerosis and septic shock [22]. One of the responses of reactive astrocytes during inflammatory process and after injury is the synthesis and release of neurotrophic factors such as nerve growth factor (NGF) [4, 6, 40]. NGF production by glial cells during such circunstances may limit the extent of neuronal loss and promote regenerative processes to restore neuronal function [28, 39]. NGF synthesis by astrocytes is a highly regulated process which includes complex interactions among different signaling pathways. Pro-inflammatory cytokines, IL-1β and TNFα are well known stimulators of NGF synthesis [14, 27, 28]. On the other hand, lipopolysaccharide, the molecule responsible for the inflammatory response during the septic shock, is also a potent inducer of NGF synthesis and secretion by astrocytes and brain macrophages [11, 21]. It has also been described that 1α,25-dihydroxyvitamin D_3 stimulates NGF synthesis and release by cultured glial cells [25]. As stated above, both cytokines and 1α,25-dihydroxyvitamin D_3 have been reported to induce ceramide accumulation [13, 26]. We have previously described the involvement of a phosphatidylcholine-phospholipase C (PC-PLC) in the regulation of NGF synthesis by astrocytes [19], which in turn has been shown to mediate TNFα activation of NF-κB through SM breakdown [30]. Therefore, it seems interesting to study the possible function of SM breakdown and ceramide release in the regulation of NGF synthesis by astrocytes.

We report here the stimulatory effect of exogenous addition of both, neutral SMase and cell-permeable ceramides on NGF synthesis and secretion in primary cultures of newborn rat astrocytes. The characteristics of this NGF regulatory pathway were studied with special emphasis on the possible effects on the MAP kinase cascade and its crosstalk with other signal transduction pathways.

2. Materials and methods

2.1. Materials

Tissue culture plastic wares and fetal calf serum (FCS) were from Nunc (Denmark). Culture media was from Biowhitakker (Belgium). Neutral SMase (S. aureus), 3-isobutyl-1-methylxanthine (IBMX) and 4β-phorbol 12β-myristate (PMA) were from Sigma Chemicals (USA). Bisindolylmaleimide GF 109203X (BIM), forskolin (FK) and cell permeable ceramide analogs were from Calbiochem (USA). ^{32}Pi and DMEM Pi free were from ICN Pharmaceuticals Inc. (USA). Anti-NGF monoclonal antibodies were from Boehringer Manheim (Germany). Anti-raf-1 polyclonal antibody was from Santa Cruz Biotechnology (USA), and anti-rabbit agarose-linked IgG from Transduction Laboratories (UK). p42/p44 MAP kinase peptide substrate and reagents for kinase assay were from Amersham (U.K.).

2.2. Primary cultures of astroglial cells

Cortical astrocytes were derived from 1-2 day old rats and cultured as previously described [19]. Cells were seeded at a density of $3x10^4$ cells/cm^2 on plastic plates previously coated with 5 μg/ml dl-polyornithine in water. Cells were cultured for 3 weeks in basal medium consisting of a mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 (1:1, v/v), with 0.66% glucose, 5 μg/ml streptomycin, 5 U/ml penicillin, and supplemented with 10% (FCS). The primary cultures consisted of 95% astrocytes as judged by immunocytochemical staining of glial fibrilary acidic protein.

Three days before the experiment, the FCS containing medium was removed and cells were transferred to a chemically-defined medium consisting of serum-free basal medium supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite.

2.3. RNA analysis and enzyme-linked immunosorbent assay (ELISA)

After the indicated times of treatment, RNA extraction was carried out according to the LiCl/urea method. Northern blot analysis was also performed by standard procedures. Glyoxal-treated RNA was fractionated in agarose gels, transferred to Hybond-N membranes by capillary blotting, and hybridized serially with a ^{32}P-labelled probe of mouse NGF cDNA. The NGF probe was the 917 bp NGF DNA cloned by Scott et al. [31].

Standardization of RNA loading was routinely controlled by hybridization of the blots with amyloid precursor protein cDNA probe [32]. Densitometric analyses were performed with PhosphorImager 445 SI (Molecular Dynamics).

For extracellular NGF determination, cell supernatants were collected 24 hours after treatment as described in the text, diluted in one volume of phosphate buffer saline (PBS) containing 0.1% Tween 20 and 0.5% gelatin, and conserved frozen until quantitation. NGF released by the cells was assayed in triplicate, by a double-site ELISA, using a monoclonal anti-NGF antibody, coupled or not to β-galactosidase according to an experimental protocol described before [19].

2.4. Raf-1 immunoprecipitation

Immunoprecipitation was performed as described previously [10]. Briefly, cells cultured in 57 cm^2 dishes, were transferred to chemically defined medium three days before the experiment. The medium was replaced by Pi-free DMEM and incubated for 4 hours with 100 μCi ^{32}Pi. Cells were stimulated as described in the text. Lysates were subsequent obtained by treating cells with a buffer containing 50mM Tris pH 7.5, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml bovine serum albumin, and 150 mM NaCl. Immunoprecipitation was carried out by incubation with 7.5 μg/ml of anti-raf-1 polyclonal antibody and precipitation with anti-rabbit agarose-linked IgG. Phosphorylation was determined in the immunoprecipitates by SDS-PAGE and autoradiography of the gels. Gels were previously stained with Coomasie blue in order to verify the loading of the gels. Autorradiographic Fuji-films were subjected to densitometric analysis using the Sigma-Gel program.

2.5. MAP kinase assay

Astrocytes were incubated for 15 min at 37°C with agents shown in Table 2, and the reaction terminated by washing with ice-cold PBS and adding 500 μl of lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 2mM EGTA, 2 mM DTT, 40 μg/ml digitonin, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, at 4°C. Cellular debris were precipitated at 25000 x g for 20 min and MAP kinase activity was measured in the supernatant . Extracts (15 μl) were then assayed by adding 10 μl of the substrate buffer (containing 6 mM substrate peptide, 75 mM Hepes, 300 μM sodium orthovanadate, and 0.05% sodium azide, pH 7.4), and 5 μl of ATP buffer (containing 0.3 mM [γ-^{32}P]ATP (300 μCi/ml) and 90 mM $MgCl_2$). After 30 min incubation at 30° C., reaction was terminated by adding 10 μl of 300 mM orthophosphoric acid. Thirty μl of each sample was spotted onto phosphocellulose discs, washed two times for 2 min in 1% acetic acid and then twice for 2 min in distilled water. Radioactivity of each disc was determined by scintillation counting.

3. Results

3.1. Enhanced synthesis and secretion of NGF by exogenous addition of neutral SMase and C_2-ceramide

In order to study the possible involvement of the SMase pathway in the regulation of NGF synthesis, primary cultures of astrocytes were exposed to increasing concentrations of neutral SMase. An increased secretion of NGF to the cell supernatant was observed at 250 mU/ml with maximal activation at 1 U/ml (15-fold increase) (Fig. 1a). Time-course experiments showed that maximal concentration of NGF on the supernatant peaked at 72 h of treatment (Fig. 2). In order to know whether the effect observed with SMase was due to the release of the second messenger ceramide, the synthetic cell-permeable analog N-acetylsphingosine (C_2-cer) was used. Treatment of cells with C_2-cer also enhanced NGF synthesis and secretion from cells, with maximal stimulation observed at 25 μM C_2-cer (Fig. 1b). The specificity of C_2-cer effect was supported by the fact that C_2-dihydroceramide, an inactive analog, had no effect on secreted NGF at the same concentrations (Table 1).

Northern blot analyses were used to test whether the content of NGF mRNA in treated cells was elicited in concert. Fig. 3 shows that after 24 h of treatment, NGF mRNA levels were higher in both SMase and ceramide-treated cells. Therefore, it seems that ceramide-induced NGF secretion is also accompanied by an increase in the mRNA levels of the neurotrophic factor. In fact, treatment of cells in the presence of actinomycin D and cycloheximide, inhibitors of DNA transcription and protein synthesis, respectively, abrogated increased levels of NGF in the cell supernatant elicited by SMase and ceramide (data not show).

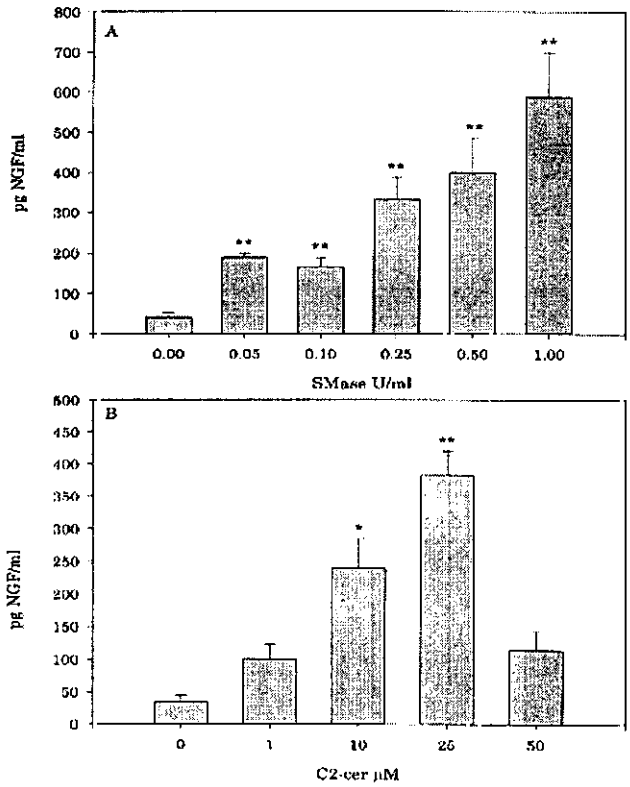

Fig. 1 Dose-response induction of secreted NGF by exogenous neutral sphingomyelinase and C_2-ceramide. Primary cultures of astrocytes were stimulated with increasing concentrations of SMase (Fig. 1a) and N-acetylsphingosine (Fig. 1b) for 24 h. Extracellular NGF was assayed with a double-site ELISA. Data are means ± S.D. from three independent experiments. Asterisks indicate significant differences from the corresponding controls (* $P< 0.05$, ** $P<0.01$, by Student's t-test).

3.2. Signaling pathways responsible for the induction of NGF secretion by ceramide

To further investigate the mechanism mediating ceramide regulation of NGF synthesis, we investigated if Ca^{2+} and diacylglycerol-dependent PKC was required for ceramide action. We have previously described that the use of bisindolylmaleimide (BIM), a potent and highly specific PKC inhibitor, completely abolished 4β-phorbol 12β-myristate (PMA) induced increase of secreted NGF [19]. Treatment of cultured astrocytes with 2 μM BIM slightly inhibited SMase-induction of NGF secretion, whereas C_2-cer-induction of NGF secretion was unaffected (Table 1). This observation points to a mainly PKC-independent mechanism for induction of NGF by ceramide.

The study of a possible involvement of the cAMP signaling pathway was undertaken using the diterpene forskolin (FK), a well known activator of adenylyl cyclase, and 3-isobutyl-1-methylxantine (IBMX), a phosphodiesterase inhibitor. FK and IBMX alone failed to increase NGF secretion (data not shown), whereas in the presence of SMase or C_2-cer they partially antagonized the stimulatory effect of the latter (40% and 60% inhibition respectively) (Table 1). Thus a regulatory crosstalk among the SMase and cAMP pathways seems to exist. The fact that intracellular cAMP raising agents have been described as inhibitors of the MAP kinase cascade [18, 35, 37], and the possible involvement of the MAP kinase cascade on the ceramide-activated signal transduction process [29], prompted us to investigate the role of the MAP kinase cascade on the ceramide-inducted stimulation of NGF production.

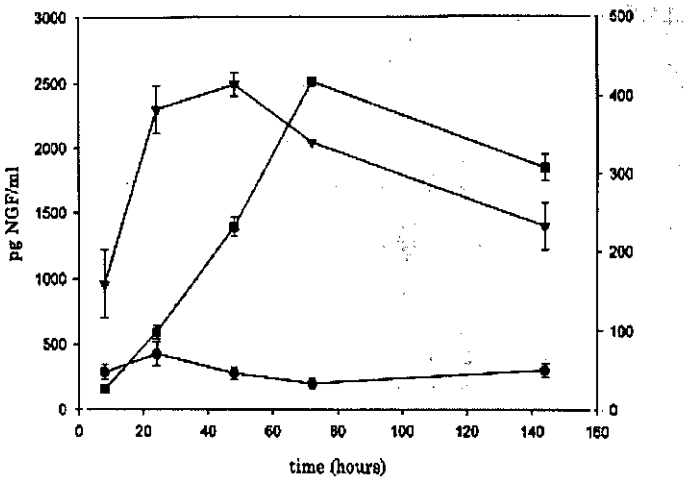

Fig. 2 Time-course of NGF secretion after treatment with exogenous neutral sphingomyelinase and C_2-ceramide. Primary cultures of astrocytes were treated with vehicle alone (●), maximal dose 1 U/ml of SMase (■)(left axis) and 25 μM C_2-cer (▼)(right axis). At the indicate times cell-secreted NGF was assayed. Data are the means ± S.E.M of two independent experiments with determinations in triplicate.

3.3. Involvement of the MAP kinase cascade in the induction of NGF secretion by ceramide

To investigate the possible role of the MAP kinase cascade in ceramide action, experiments with PD098059 or apigenin were conducted. PD098059 has been described as a selective inhibitor of MAP kinase kinase (MEK) [8]. It binds to unactivated MEK preventing its activation by raf-1 or MEK kinase [1]. Apigenin is an inhibitor of the MAP kinase cascade, probably by acting at the raf-1 kinase level [17]. Treatment of primary astrocytes with 100 μM PD098059 abolished the induction of NGF secretion elicited by SMase and C_2-cer (Table 1). The dose-response curve of inhibition showed an IC_{50} around 20 μM (Fig. 4A). Treatment of cells with 25 μM apigenin prevented the SMase and C_2-cer inducted secretion of NGF (65% and 85% inhibition respectively) (Table 1), the IC_{50} value is approximately 10 μM (Fig. 4B). Therefore, data indicate that the MAP kinase cascade may be implicated in the regulation of NGF synthesis by SM hydrolysis.

3.4. Ceramide increases the phosphorylation extent of raf-1 and MAP kinase activity

To further study the intracellular effects of ceramide, experiments were undertaken to study the phosphorylation state of raf-1. Immunoprecipitation of raf-1 from ^{32}P-labelled cells is shown in Fig. 5. Short treatment of astrocytes with SMase lead to an increased phosphorylation of raf-1, whereas C_2-cer failed to induce raf-1 phosphorylation. In order to asses the role of endogenous ceramides in raf-1 regulation we employed the more physiological ceramide analog N-octanoylceramide (C_8-cer). C_8-cer treatment of cells resulted in an enhanced phosphorylation of raf-1, similar to that elicited by SMase (Fig. 5). SMase and C_8-cer-induced phosphorylation was still detectable after 30 minutes of stimulation (data not shown). Changes in the phosphorylation state of raf-1 will result changes in its kinase activity. This observation thus confirms previous reports about the role of ceramide in raf-1 phosphorylation [2, 38]

MAP kinase activity was meassured as phosphorylation levels of a highly selective peptide substrate for p42/p44 MAP kinase [5]. Astrocyte treatment with C_2-cer, C_8-cer and SMase evoked MAP kinase activation (Table 2). Activation elicited by SMase was higher than that elicited by both ceramide analogs. Specificity of MAP kinase activation was corroborated by the lack of effect of the inactive C_2-dihydroceramide (Table 2). Moreover pretreatment with the MAP kinase cascade inhibitor

Table 1

Addition	pg NGF/ml			
None	31.7	±	8.9	
SMase	588.0	±	66.4	(n=5) a
BIM + SMase	509.4	±	43.5	(n=3) a
FK + SMase	369.3	±	37.0	(n=3) a, b
IBMX + Smase	355.7	±	34.7	(n=3) a, b
API + SMase	205.5	±	32.7	(n=3) a, b
PD 098059 + Smase	60.7	±	17.0	(n=3) b
C_2-Cer	383.0	±	46.8	(n=4) a
BIM + C_2-cer	363.2	±	40.3	(n=3) a
FK + C_2-Cer	154.7	±	67.8	(n=4) a, c
IBMX + C_2-Cer	157.4	±	44.4	(n=4) a, c
API + C_2-Cer	62.8	±	19.9	(n=4) c
PD 098059 + C_2-Cer	43.8	±	5.6	(n=3) c
C_2-dihydrocer	35.0	±	17.0	(n=3)

Table 1. Effect of different agents in sphingomyelinase and C_2-cer induction of NGF secretion. Primary cultures of astrocytes were incubated with the indicated agents for 24 h. 2 μM BIM, 10 μM FK, 100 μM IBMX, 25 μM API and 100 μM PD 098059 were added 1 hour prior to 1 U/ml SMase or 25 μM C_2-cer stimulation. Data are the means ± S.D. of the number of independent experiments indicated in each case. Statistical analysis was performed by Student's t-test, significant differences are indicated: a, $P<0.01$ versus incubations with no addition; b, $P<0.01$, versus incubations with SMase alone; c, $P< 0.01$, versus incubations with C_2-cer alone).

Fig. 3 Northern blot analysis of NGF mRNA accumulation. Lane 1, untreated cells; lane 2, 1 U/ml SMase; lane 3, 25 μM C_2-cer. After 24 h incubation of Northern blot was performed as described in Matherial and Methods with NGF and APP cDNA probes. Essentially identical results were obtained in three independent experiments.

PD098059,.counteracted ceramide induction of MAP kinase activity (Table 2). These results support the notion that signal transduction triggered by SM hydrolysis may activate MAP kinase and point to a role of the MAP kinase cascade in the regulation of NGF secretion by the SM pathway.

4. Discussion

The present report shows that exogenous addition of neutral SMase or C_2-cer, a cell permeable analog of ceramide, induces a dose-response and time-dependent stimulation of NGF synthesis and secretion. The effect of added SMase indicates that endogenously produced ceramides may also be able to induce NGF synthesis and corroborates the specificity of the ceramide analog action. The fact that increased

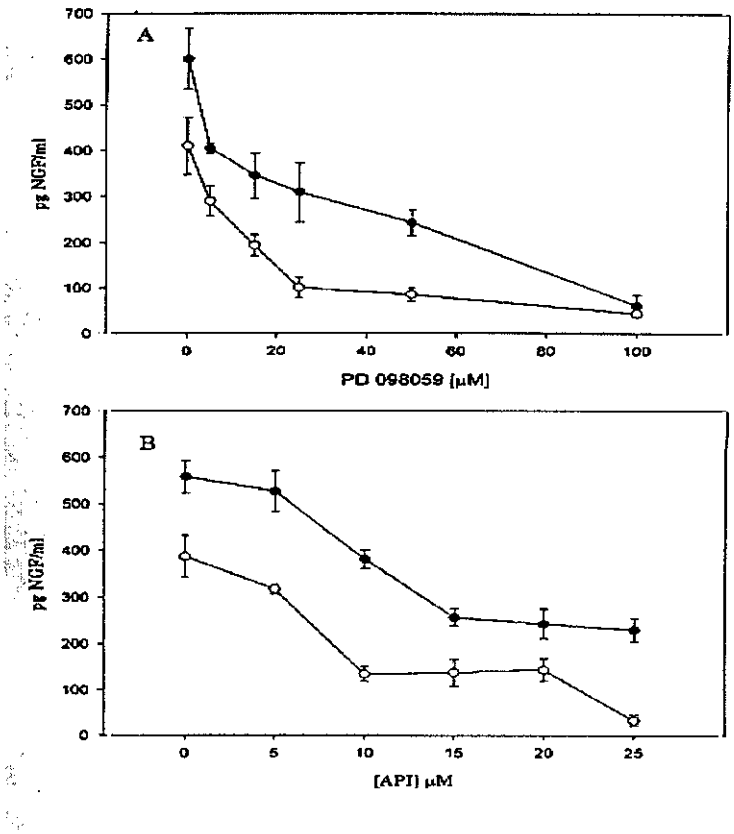

Fig. 4 Dose-response inhibition by PD 098059 and apigenin of sphingomyelinase and C_2-ceramide induction of NGF secretion. Primary cultures of astrocytes were incubated with 1 U/ml SMase (●) and 25 μM C_2-cer (○) and the indicated doses of PD 098059 (A) or apigenin (B) for 24 h., and secreted NGF subsequent assayed. Data are the means ± S.E.M. of two independent experiments, with determinations in triplicate.

NGF protein secretion to the cell supernatant was accompanied by an increase in NGF mRNA levels, together with the data obtained with transcription and translation inhibitors, supports the notion that increased NGF output is the result of an augmented synthesis of NGF.

Regulation of NGF production by a number of agents in primary astrocytes has been shown to be dependent on PKC activation [7, 24]. However, IL-1β and vitamin D_3 induce NGF production by mechanisms independent of PKC activity [25, 28]. These agents are also well known to exert some of their effects through the SM pathway and the generation of ceramide [13, 26, 33]. It is worth noting that lipopolysaccharide, which is able to mimic some of the effects of ceramide [16, 36] is also a PKC-independent inductor of NGF secretion [11]. We have previously reported that PC-PLC is able to enhance NGF synthesis by primary astrocytes by a signaling mechanism which is only partially dependent of PKC [19]. PC-PLC action, may in turn be coupled to ceramide generation [30]. In fact, recent results show that short treatment of C6 glioma cells with PC-PLC lead to the translocation and phosphorylation of PKC ζ [10], which is one of the molecular targets of ceramide [20]. Our results indicate that ceramide induction of NGF in primary astrocyte cultures is independent of PKC. Hence the SM pathway may represent one of the main PKC-independent signaling pathways leading to the regulation of NGF production by glial cells. This is in line with previous reports showing the involvement of the SM pathway in the induction of IL-6 synthesis, in human astrocytoma cells [9]. Raf-1 is a member of the MAP kinase cascade that is activated upon cellular stimulation with a number of effectors as pro-inflammatory cytokines [2]. The precise mechanism by which raf-1 is

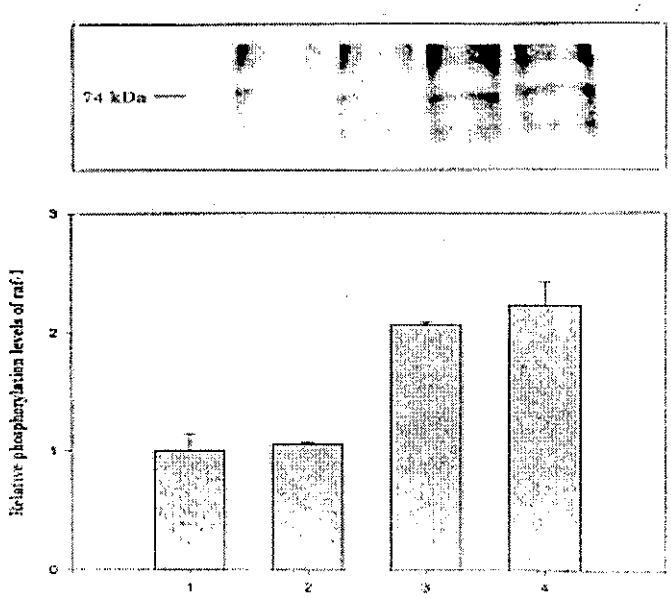

Fig. 5 Immunoprecipitation of raf-1. ^{32}P-labelled cells were stimulated for 15 minutes with the indicated agents and raf-1 was, subsequently immunoprecipitated with a polyclonal anti-raf-1 antibody. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. Densitometric data are means ± S.E.M. of two independent experiments.

Table 2

Addition	pmol/min/mg
None	98.6 ± 22.6
SMase	555.5 ± 17.7 a
C_2-cer	232.9 ± 11.8 a
C_8-cer	290.1 ± 2.0 a
C_2-dihydrocer	103.6 ± 5.9
PD 098059 + C_2-Cer	125.3 ± 9.6 b
PD 098059 + C_8-Cer	155.9 ± 8.4 c

Table 2. Effect of ceramide analogs and sphingomyelinase on MAP kinase activity. Primary cultures of astrocytes were incubated with 1 U/ml SMase, 25 μM C2-cer, or 25 μM C8-cer for 15 min. 100 μM PD 098059 was added 1 hour prior to stimulation. Data, expressed as pmol phosphate transferred /min/mg protein, are the means ± S.D. of three independent experiments performed in triplicate. Statistical analysis was performed by Student's t-test, significant differences are indicated: a, $P<0.01$ versus incubations with no addition; b, $P<0.01$, versus incubations with C_2-cer alone; c, $P< 0.01$, versus incubations with C_8-cer alone).

activated is as yet uncertain, but it is generally agreed that changes in its state of phosphorylation produce changes in its kinase activity. Recent reports indicate that raf-1 may be phosphorylated by TNF-activated CAPK, increasing its activity towards MEK [38]. Therefore it seems that the SM pathway could be linked with the MAP kinase cascade, probably at the level of raf-1, through the activation of the CAPK [2, 29, 38]. Our results indicate that the regulation of NGF synthesis by SMase and ceramide may depend on the activation of the MAP kinase cascade. It should be noted that SMase-

induction of NGF secretion was less sensitive to MAP kinase inhibition than C_2-cer-induction of NGF secretion, in concordance with its greater effect on MAP kinase activity.

It has been described that hyperphosphorylation of raf-1 induced by intracellular cAMP-raising agents leads to the blockade of the MAP kinase cascade [37]. A similar inhibitory effect of the MAP kinase cascade by cAMP-increasing agents has also been described in astroglial cells stimulated with growth factors and LPS [18, 35]. Results in the present report show that treatment with FK and IBMX abolishes half of the ceramide action on NGF production by astrocytes. Thus, the existence of an inhibitory crosstalk between the SM and the cAMP pathways appears to exist also in the regulation of NGF synthesis by ceramide.

The possible involvement of the MAP kinase cascade as the signaling mechanism underlying ceramide generation is enforced by the observed increase in raf-1 phosphorylation and MAP kinase activity as the result of short-term treatment with SMase or ceramide analogs. Short-chain ceramide analogs are supposed to be biologically more active than their long chain equivalents likely due to the increased solubility of the former. However, long chain ceramides have been described to be more potent than short chain ceramides in activating raf-1 [15].This is in concordance with our results showing an increased phosphorylation state of raf-1 from C_8-cer-stimulated treatment of cells, whereas C_2-cer failed to induce such effect. Phosphorylation of raf-1 by the ceramide analog C_8-cer has been described also previously by other authors [38]. Thus, it seems plausible that ceramide action on NGF expression may be mediated by activation of the MAP kinase cascade at the level of raf-1 and p42/p44 MAP kinase.

In conclusion, we report here the role of the SM pathway in the regulation of NGF synthesis and secretion by cultured rat astrocytes. The action of ceramide on NGF production seems to be partially mediated by activation of the MAP kinase cascade. The regulatory action of ceramide may be related to previously described effects of pro-inflammatory cytokines such as IL-1β and TNFα, and is therefore of maximal importance in our understanding of the processes occurring during inflammatory brain lesions and certain neuropathological disorders.

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RESULTADOS Y DISCUSIÓN

Regulación de la secreción de NGF por la PC-PLC

El estudio de la acción de la PC-PLC en la regulación de la síntesis de NGF se justifica por dos importantes razones. Resultados preliminares pusieron de manifiesto la importancia de la acción de esta fosfolipasa C en relación a otras de diferente especificidad por el substrato. Así, aunque la PI-PLC y PLD tienen un efecto inductor en la secreción de NGF, este es cinco veces menor que el mostrado por la PC-PLC. En este sentido, el sistema de transducción de señales acoplado a la hidrólisis de fosfoinosítidos, presente en astrocitos en cultivo primario, no parece ser uno de los mecanismos de regulación de expresión del NGF (Jehan et al., 1995). A estas observaciones se suma el hecho de que la hidrólisis de PC podría ser importante en la generación de un aumento de larga duración en los niveles intracelulares de DAG (Asaoka et al., 1996; Nakamura et al., 1996). Este efecto de la hidrólisis de PC permitiría explicar ciertos mecanismos de regulación celular a largo plazo como el crecimiento, la diferenciación celular, o la expresión del gen de NGF, considerado de expresión tardía.

La inducción de NGF por la acción de la PC-PLC exógena es parcialmente dependiente de la actividad de cPKCs, dependientes de Ca^{2+} y DAG, como pone de manifiesto el efecto inhibidor de la bisindoleilmaleimida GF 109203X (BIM). Este efecto coincide con el observado al disminuir los niveles de PKC por exposición prolongada de los astrocitos a ésteres de forbol ("downregulation"). Se observa además que la SF, producto del metabolismo de las ceramidas por una ceramidasa (Nikolova-Karakashian et al., 1997), es capaz de inducir la secreción de NGF en igual extensión que la PC-PLC en células preincubadas con BIM o con los niveles de PKC disminuidos por el efecto de "downregulation" causado por ésteres de forbol. Estas observaciones coinciden con evidencias experimentales en otros sistemas celulares, que implican a la PC-PLC como posible activador de la vía de señalización de la SMasa-ceramida, responsable de la generación de ceramidas (Schütze et al., 1992; Müller et al., 1994; Wiegman et al., 1994). Se plantea por tanto la hipótesis de que la acción de la PC-PLC podría activar la síntesis de NGF por un doble mecanismo: generación de DAG y activación de cPKCs, y por otro activación de la hidrólisis de esfingolípidos.

Para estudiar el efecto de la vía de la SMasa-ceramida en la síntesis de NGF utilizamos el análogo permeable de ceramida, C2-ceramida y el tratamiento con SMasa exógena de *Staphylococcus aureus*. La estructura de las ceramidas permeables

con una cadena acilada más corta facilita el paso de estos compuestos al interior celular (Biewlaska et al., 1992). Por otra parte el tratamiento con SMasa exógena genera ceramida endógena por la hidrólisis de la SM situada en la cara externa de la membrana plasmática (Olivera et al., 1992). Aunque se ha descrito la existencia de ciertas diferencias en las respuestas inducidas por estos análogos y las ceramidas de origen endógeno (Andrieu et al., 1996; Zhang et al., 1997), la utilización de este tipo de aproximación experimental se considera válida para el estudio de la regulación de numerosos procesos celulares por la vía de señalización de la SMasa-ceramida (Fiebich et al., 1995; Yao et al., 1995; Casaccia-Bonnefil et al., 1996; Cuvillier et al., 1996; Zhang et al., 1997).

Regulación de la secreción de NGF por diferentes componentes de la vía de la SMasa

Uno de los primeros interrogantes que se plantean al estudiar el papel de la vía de la SMasa-ceramida en la regulación de la síntesis del NGF es el de determinar cual o cuales de sus intermediarios pueden ser responsables del efecto regulador de la síntesis de NGF. La SF induce un aumento de aproximadamente cinco veces en los niveles extracelulares de NGF respecto de las células control. Sin embargo, el derivado fosforilado, SF-1-P, potencial mediador activo de la SF (Spiegel et al., 1997) carece de efecto en la secreción de NGF (resultados no incluidos). Por otro lado, las ceramidas son inductores significativamente más potentes de la secreción de NGF que la SF. Estas observaciones hacen suponer que el efecto de la SF podría estar mediado por su paso a ceramida (Goldkorn et al., 1991), sin descartar un efecto directo de la propia SF.

La diferencia observada entre ceramida y SF en la regulación de la síntesis de NGF, supone una aportación al debate sobre la significación biológica que tiene la inducción de NGF por los mensajeros derivados de esfingolípidos. Así, se ha postulado que mientras que la generación de ceramida es responsable de respuestas biológicas como la diferenciación celular, supresión del crecimiento o apoptosis (revisado por Hannun, 1996; Testi, 1996), la SF y SF-1-P parecen ser inductores de respuestas mitogénicas (Olivera y Spiegel, 1993; Spiegel et al., 1997). En este sentido, se ha propuesto recientemente que citoquinas pro-inflamatorias y factores de crecimiento favorecen la acumulación de uno u otro tipo de mensajeros en función de la diferente regulación que ejercen sobre la actividad SMasa, ceramidasa, y SF quinasa (Coroneos et al., 1995; Nikolova-Karakashian et al., 1997), de manera que el balance entre los dos tipos de esfingolípidos podría regular el equilibrio entre diferenciación/muerte

celular y división/proliferación celular. Se ha demostrado recientemente que, en efecto, la SF-1P es capaz de revertir la muerte celular programada inducida por ceramida (Cuvillier et al., 1996). Si este esquema es válido en células gliales, la síntesis de NGF estaría asociada a los procesos de diferenciación celular e inhibición del crecimiento, y por tanto estimulada y coordinada con respuestas de estrés celular.

La estimulación por ceramidas no resulta aparentemente citotóxica

La generación de ceramidas además de inducir apoptosis en numerosos sistemas celulares, parece ser uno de los mecanismos responsables de la citotoxicidad de citoquinas pro-inflamatorias como el TNFα (Hannun, 1996; Testi, 1996). Por todo ello, es importante comprobar que la incubación de las células con los diferentes moduladores utilizados no es citotóxica. Esto se comprobó cualitativamente dada la ausencia de células despegadas del soporte de cultivo después de los tratamientos de 24 horas. Además, se analizó la viabilidad celular tras las incubaciones mediante el "ensayo de MTT", basado en la reducción del anillo de tetrazolio por la actividad de las deshidrogenasas mitocondriales. En el intervalo de concentraciones utilizado de C2-ceramida sólo las células tratadas con C2-ceramida 50 μM presentan una menor viabilidad celular, que coincide con una disminución en los niveles de NGF extracelulares. La ausencia de efecto citotóxico de las ceramidas sobre cultivos primarios de astrocitos, concuerdan con resultados de otros autores que evidencian que los astrocitos son resistentes a la muerte celular inducida a través de la ruta de la SMasa-ceramida (Casaccia-Bonnefil et al., 1996a;b).

Regulación de la síntesis y secreción de NGF por el aumento en los niveles intracelulares de ceramida

Los resultados expuestos ponen de manifiesto un significativo aumento en la producción de NGF inducido por el análogo permeable de ceramida, C2-ceramida, así como por acción de SMasa exógena. La inducción de NGF por la generación de ceramidas es independiente de la activación de cPKCs dependientes de Ca^{2+} y DAG, como pone de manifiesto la ausencia de inhibición por BIM. La inducción de la síntesis de NGF estimulada por ceramidas se inhibe parcialmente por el aumento de los niveles de cAMP. El efecto inhibidor del cAMP sobre la inducción de NGF coincide con el observado para otros inductores de NGF como el suero, PMA o TGF-β1 (Jehan et al., 1993;1995; Han et al., 1994).

Implicación de la cascada de MAPK en la síntesis de NGF inducida por ceramidas

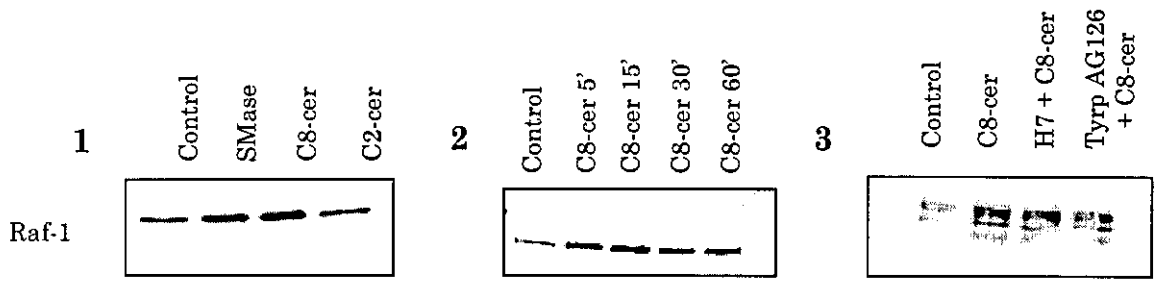
Los resultados obtenidos muestran como la secreción de NGF inducida por ceramidas se inhibe por apigenina y el inhibidor sintético PD 098059, lo que sugiere que la inducción depende de la activación de la cascada de MAPK (Alessi et al., 1995). En efecto, tanto los análogos permeables de ceramida como la SMasa exógena incrementan la actividad p42/p44 MAPK en cultivos primarios de astrocitos, confirmándose la especificidad de esta activación por el inhibidor PD 098059. El estudio detallado de la cinética de activación de MAPK por C2- y C8-ceramida mostró que en todos los tiempos ensayados la activación de MAPK es mayor en células tratadas con C8-ceramida que con C2-ceramida (datos no incluidos). Estas observaciones confirman que los análogos de ceramida de cadena acilada más larga son mejores activadores de Raf-1 (Huwiler et al., 1996). La activación de MAPK por ceramidas se mantiene al menos durante 60 minutos, un efecto sostenido que apoya un posible papel de este sistema de fosforilación en la regulación celular a medio y largo plazo, como la síntesis de NGF.

El papel de la activación de MAPK por ceramidas para explicar algunos de sus efectos biológicos como la apoptosis, es objeto de controversia (Sasaki et al., 1995; Coroneos et al., 1996; Pyne et al., 1996; Brenner et al., 1997). Sin embargo, es de destacar que los análogos permeables de ceramida inducen un aumento de la actividad MAPK que es del mismo orden de magnitud que la activación de MAPK producida por otros inductores de la síntesis de NGF como TNFα o LPS (Mallat et al., 1989; Hattori et al., 1996).

Estudio detallado del posible mecanismo de activación de MAPK por ceramidas

La activación de MAPK por ceramidas puede explicarse a través del efecto activador de estos lípidos sobre Raf-1 (Yao et al., 1995; Huwiler et al., 1996). La estimulación de los astrocitos con C8-ceramida o SMasa, produce un aumento en los niveles de fosforilación de la proteína Raf-1 inmunoprecipitada, lo que explicaría la activación observada de MAPK. Puesto que la activación de Raf-1 se asocia con una translocación a membrana plasmática (Stokoe et al., 1994), se estudió la posible translocación de Raf-1 tras la estimulación por ceramidas. Como se muestra en la figura de la siguiente página, el tratamiento con ceramidas (SMasa exógena 1 U/ml o análogos permeables 25 μM) durante 15 min aumenta los niveles de proteína en fracción particulada. El efecto de la C8-ceramida es de nuevo de mayor intensidad que el obtenido con la C2-ceramida, lo que coincide con los resultados previamente

descritos. La translocación de Raf-1 a membrana inducida por C8-ceramida ocurre muy rápidamente, desde los primeros 5 minutos de estimulación, y se mantiene al menos durante los primeros 60 min. La translocación y activación de Raf-1 va acompañada de la fosforilación en tirosina y serina/treonina (Morrison y Cutler, 1997). La fosforilación en tirosina es uno de los marcadores característicos de activación de la proteína dependiente de Ras (Marais et al., 1995) y la fosforilación en serina/treonina puede estar mediada por PKC o por la propia proteína quinasa activada por ceramida CAPK (Yao et al., 1995; Morrison y Cutler, 1997). En la figura inferior se muestra como el aumento de los niveles de fosforilación de Raf-1 en células marcadas con ^{32}P, también se pone de manifiesto con un anticuerpo específico para tirosina fosforilada. La especificidad del marcaje se demuestra por la ausencia de efecto del inhibidor H7 de serina/treonina quinasas, mientras que el inhibidor de tirosina quinasa, tirfostina AG126, revierte parcialmente la fosforilación.



Regulación de Raf-1 por ceramidas. 1/ Translocación de Raf-1 a la fracción particulada en cultivos primarios de astrocitos tratados durante 15 min con SMasa (S.aureus) 1 U/ml, C8- y C2-ceramida 25 μM. 2/ Cinética de la translocación de Raf-1 inducida por C8-ceramida 25 μM. 3/ Inmunoprecipitación de la proteína Raf-1 revelada con un anticuerpo policlonal anti-fosfotirosina. Las células se estimularon con C8-ceramida 25 μM durante 15 min y donde se indica preincubadas durante 1 h. con H7 o tirfostina AG126 100 μM. Los resultados de la figura corresponden a un experimento representativo de otros 3 o 5 realizados y se obtuvieron mediante los procedimientos descritos en las publicaciones incluidas en la presente memoria.

2. REGULACIÓN DE LA PKC ζ POR MENSAJEROS LIPÍDICOS EN CÉLULAS GLIALES

En este capítulo se estudia el posible papel modulador de diferentes mensajeros lipídicos sobre la isoforma ζ de PKC atípica. La PKC ζ está relacionada con la activación de la cascada de MAPK en diferentes modelos experimentales (Berra et al., 1995; Liao et al., 1997). Por otro lado, las ceramidas ejercen un efecto regulador de la actividad de PKC ζ, que es responsable de algunas de las respuestas celulares de la vía de la SMasa-ceramida como la activación del factor de transcripción NF-κB (Lozano et al., 1994; Müller et al., 1995). Estas observaciones sugieren la posibilidad de que la proteína PKC ζ este implicada en el sistema de transducción de señales iniciado por la PC-PLC, mediando la activación de Raf-1 y cascada de MAPK por ceramidas, y regulando en ultima instancia la síntesis de NGF.

Primeramente se expone el efecto de la actividad de la PC-PLC exógena sobre la PKC ζ en la línea celular de glioma C6 y, posteriormente, se estudia el efecto que en esta misma proteína, PKC ζ, tienen los análogos permeables de ceramida y la SMasa exógena.

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Addition of phosphatidylcholine-phospholipase C induces cellular redistribution and phosphorylation of protein kinase C ζ in C 6 glial cells

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Abstract

Phosphatidylcholine breakdown has been shown to play a critical role in signal transduction involving generation of a number of second messengers [Exton, J.H., Biochim. Biophys. Acta, 1212 (1994) 26-42]. In the present report we demonstrate by immunofluorescence that short-treatment of C 6 glial cells with phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC), changes the intracellular localization of protein kinase C (PKC) ζ from the cytoplasm to a perinuclear region. Western blot analysis also showed a redistribution of PKC ζ after incubation of cells with PC-PLC. To test whether these changes were accompanied by an activation of the enzyme, we measured the extent of phosphorylation of PKC ζ by immunoprecipitation from ^{32}P-labelled cells. Short-treatment with PC-PLC resulted in enhanced phosphorylation of the higher Mr PKC ζ in C 6 glial cells.

Keywords: Protein kinase C ζ; Phosphatidylcholine-phospholipase C; Glial cells

Protein kinase C (PKC) is one of the major mediators of signals upon external stimulation of cells by hormones, neurotransmitters, and growth factors. PKC has been found in most mammalian tissues, but is particularly abundant in the brain [17]. The mammalian PKC polypeptides identified to date by cDNA cloning have been classified into three groups based on two criteria, namely structural features and regulation by cofactor. The atypical PKCs (ζ and ι/λ isoforms) are dependent on phosphatidylserine, but do not require Ca^{2+} or diacylglycerol for activation [4,13].

In astrocytes and glial cells the presence of PKC α, β, γ, δ, ε and ζ isoforms has been reported [5,7,11,14]. Profiles of PKC isoforms in rat astrocytes and C 6 glioma cells are similar, although a higher expression of α, ε, and ζ isoforms in glioma cells has been detected [14]. Recently, it has been demonstrated that the PKC ζ isoform plays an important role in mitogenic signal transduction [1], neuronal differentiation [19], and maintenance of long-term potentiation [15]. However, the precise mechanism by which PKC ζ activity is regulated is not fully understood. PKC ζ is not activated by diacylglycerol or phorbol esters [13,18] and in many cell types including glial cells, PKC ζ is neither translocated from cytosol to membrane nor down-regulated in response to acute or chronic treatment with phorbol esters [2]. Activation of PKC ζ may be achieved by ceramide [10]. The second messenger ceramide is generated by the action of sphingomyelinase, which is in turn functionally coupled to phosphatidylhydrolyzing phospholipase C (PC-PLC) [16].

We have recently shown that PC-PLC enhances nerve growth factor synthesis in cultured glial cells by a mechanism which is partially independent of the classical isoforms of PKC [8]. In the present report we show that addition of PC-PLC induces cellular redistribution and phosphorylation of PKC ζ in C 6 glial cells.

To test whether PC-PLC could have an effect on PKC ζ we analyzed the intracellular localization and the extent of phosphorylation of this PKC isoform. Short-term-treatment of C 6 cells with exogenous PC-PLC produced a change in the localization of PKC ζ from cytoplasm to a perinuclear membrane region (Fig. 1A,B). Non-specific labeling was shown after incubation with anti-PKC ζ antibody in the presence of a competing peptide used to rinse the antibody (Fig. 1C).

As a further proof of the effect of PC-PLC on the localization of PKC ζ, particulate, and soluble fractions of cells were separated by centrifugation, and PKC ζ was detected

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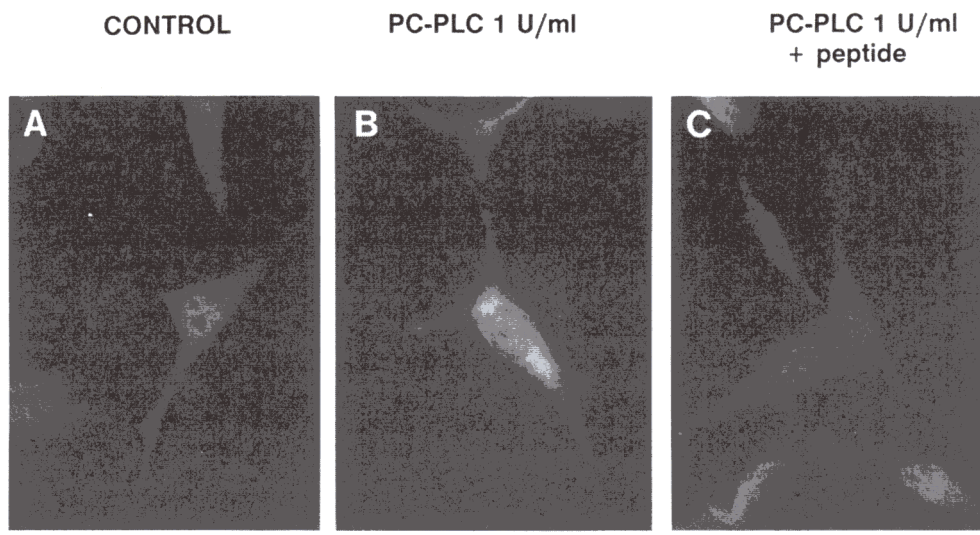

Fig. 1. Immunocytochemical localization of PKC ζ in C 6 glial cells. Cells were seeded (104 cells/cm^2) in glass coverslips, grown in serum-containing medium, and made quiescent by serum starvation. Then, no addition (A) or 1 U/ml PC-PLC from *Bacillus cereus* (B,C) were added for 15 min. Immunolocalization was performed as in [3] and coverslips were incubated with an anti-PKC ζ antibody (Life Technologies, Inc.) (A,B) or with the anti-PKC ζ antibody in the presence of the competing peptide (C). Identical results were obtained in three independent experiments.

by immunoblotting. PKC ζ migrated as two distinct bands, one corresponding to the 'embryonic form' (75 kDa) and another corresponding to the 'adult form' (83 kDa). Both forms have been detected in cultured glial cells [14]. In cells shortly treated with PC-PLC, an increase in the total amount of PKC ζ bound to the particular fraction versus control was observed (from 1828 ± 161 to 2479 ± 193 densitometric units). A decrease in total soluble PKC ζ of an equal extent (from 1470 ± 97 to 931 ± 102 densitometric units) was also detected (Fig. 2). This observation indicates that when cells are treated with PC-PLC, an association of PKC ζ to a membrane structure occurs. This translocation affected mainly the 83 kDa form, which may indicate the existence of different regulatory properties of both isoforms. As may be inferred from immunofluorescence labeling (Fig. 1), this structure seems to be located in a perinuclear region. Previous reports showed in another system the intimate association of PKC ζ with a perinuclear region of the cells [9]. As brain PKC ζ activity is significantly dependent on phospholipids [13], the translocation of the enzyme from the cytoplasm to perinuclear region may indicate an activation process.

Activation of PKC is regulated by phosphorylation, which reflects the activity state of the kinase [6]. In

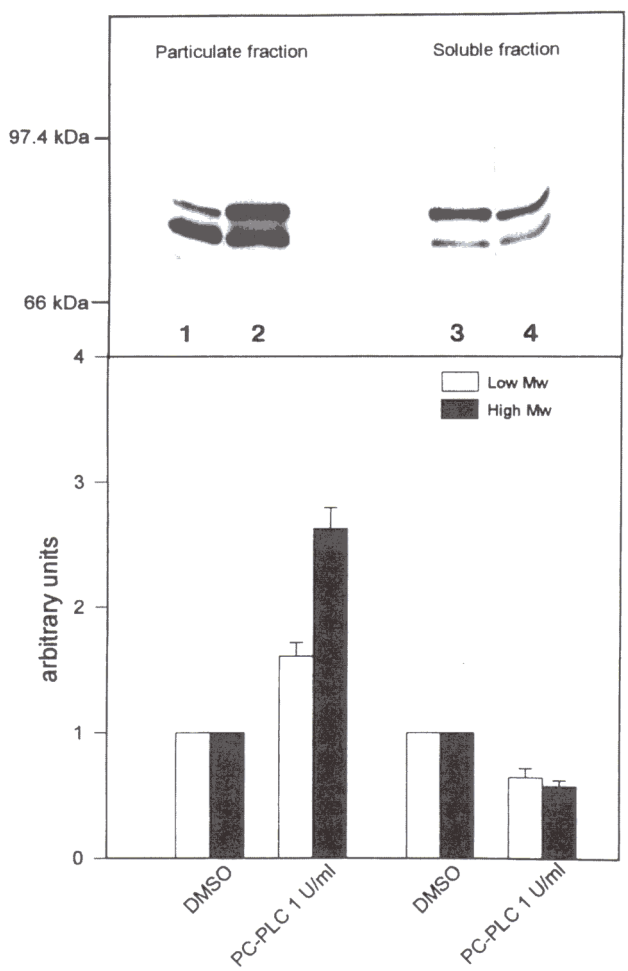

Fig. 2. Identification of PKC ζ by immunoblotting. C 6 cells were either untreated (lanes 1 and 3) or treated for 15 min with 1 U/ml PC-PLC (lanes 2 and 4). Then, particulate and cytosolic fractions were obtained from cell lysates by centrifugation at 50 000 *g* and resolved by electrophoresis. The markers on the left correspond to the migration position of 97.4 and 66 kDa molecular weight markers. Luminograms obtained with the ECL detection kit were scanned with a Scanjet 4c/T (Hewlett Packard). Densitometric analyses were performed with the computer program Sigma Gel 1.0 (Jandel Scientific). Arbitrary units are related to densitometric units from control cells. Identical results were obtained in three independent experiments.

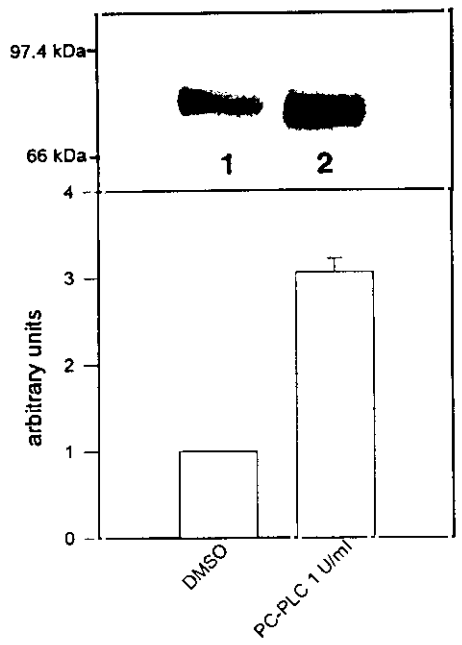

Fig. 3. Phosphorylation level of PKC ζ. C 6 cells were incubated with ^{32}P-orthophosphate (Amersham) and then either untreated (lane 1) or treated for 15 min 1 U/ml PC-PLC (lane 2). After immunoprecipitation with an anti-PKC ζ antibody, pellets were resolved by electrophoresis and subjected to autoradiography. Densitometric analysis was performed as described for Western blot. The markers on the left correspond to the migration position of 97.4 and 66 kDa molecular weight markers. Identical results were obtained in three independent experiments.

order to know whether translocation of PKC ζ correlates with an activation of the enzyme, we determined the extent of phosphorylation of PKC ζ by immunoprecipitation from ^{32}P-labeled C 6 cells. Thus, incubation of ^{32}P-labeled cells with PC-PLC led to an enhanced phosphorylation of PKC ζ (Fig. 3). Increased phosphorylation of PKC ζ affected one form, probably corresponding to 83 kDa, which agrees with its observed translocation. A similar effect has been demonstrated in epidermal cells, where phorbol myristate acetate-treated cells showed an increased phosphorylation affecting to higher-Mr PKC ζ isoform [12].

Taken together these results indicate that treatment of C 6 cells with PC-PLC causes a change in the intracellular localization of PKC ζ which becomes associated to a perinuclear region. Redistribution of this kinase was accompanied by an increase in the extent of phosphorylation of the enzyme, confirming the hypothesis that PC-PLC treatment of C 6 glioma cells leads to the translocation and consequent activation of PKC ζ isoform.

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Ceramide-induced translocation of protein kinase C ζ in primary cultures of astrocytes

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Abstract **The present research was undertaken to study the possible involvement of the atypical protein kinase C (PKC) ζ in ceramide signal transduction in primary cultures of rat astrocytes. As shown by Western blot analysis, translocation of immunoreactive PKCζ to the particulate fraction occurred upon exposure of astrocytes to cell-permeable ceramide analogs or to exogenous sphingomyelinase. The particulate fraction may correspond to a perinuclear area, as indicated by immunocytochemical techniques. Furthermore, treatment of cells with *N*-octanoylsphingosine led to an increased phosphorylation of PKCζ. Results thus show that stimulation of PKCζ may be one of the intracellular events triggered by activation of the sphingomyelin pathway.**

Key words: Protein kinase C ζ; Ceramide; Sphingomyelin; Astrocyte

1. Introduction

Sphingolipids are currently recognized as active participants in the regulation of a wide range of cellular responses such as regulation of cell growth, differentiation, transformation and death [1]. Sphingomyelin (SM) breakdown occurs in multiple cell types in response to a variety of extracellular mediators including cytokines e.g. tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interferon-γ and other agents such as nerve growth factor (NGF), 1α,25-dihydroxyvitamin D_3, Fas ligand and ionizing radiation (reviewed in [1–3]). Receptor-mediated hydrolysis of SM generates ceramide, which may function as a second messenger activating several serine/threonine protein kinases and phosphatases [4]. Among the former, one of the atypical isoforms of protein kinase C (PKC), PKCζ, has been proposed as an intracellular target of ceramide action [5,6]. These atypical PKC members (ζ and λ/ι) are dependent on phosphatidylserine but are not affected by diacylglycerol, phorbol esters or Ca^{2+} [7,8]. Although PKCζ has been shown to be involved in the control of a number of cellular functions including mitogenic signal transduction [9], neuronal differentiation [10] and long-term potentiation [11], the signal mechanisms downstream PKCζ activation are not well known. PKCζ has been suggested to play a role in the activation of the mitogen-activated protein kinase (MAPK) pathway [12]. PKCζ has also been shown to be involved in the activation of nuclear factor κB (NF-κB), which is a landmark of the TNFα mechanism of action. Little is known about the specific signal transduction pathways linking TNF receptors to NF-κB. A connection has been proposed between TNFα receptor and phosphatidylcholine phospholipase C (PC-PLC), which is coupled to sphingomyelinase (SMase), resulting in the generation of ceramide [6,13,14]. We have recently demonstrated that PC-PLC induces cellular redistribution and phosphorylation of PKCζ in glial cells [15]. The present work was thus undertaken to test whether PKCζ may also be a target for ceramide action in primary cultures of astrocytes.

2. Materials and methods

2.1. Materials

Anti-PKCζ antibody, raised against amino acids 577–592 of rat PKCζ, was from Life Technologies Inc. (Gaithesburg, MD, USA). Fluorescein-conjugated donkey anti-rabbit IgG was from Amersham (Little Chaltfont, UK). D-erythro-*N*-acetylsphingosine (C_2-ceramide), D-erythro-*N*-octanoylsphingosine (C_8-ceramide) and D-erythro-dihydro-*N*-acetylsphingosine (DHC) were from Calbiochem (La Jolla, CA, USA). Protein A-agarose was from Transduction Laboratories (Lexington, KY, USA). Neutral SMase (*S. aureus*) and all other reagents were from Sigma Chemicals (St Louis, MO, USA).

2.2. Primary cultures of astrocytes

Cortical astrocytes were derived from 1–2 day old rats and cultured as previously described [16]. Cells were seeded at a density of 3×10^4 cells/cm^2 on plastic plates previously coated with 5 μg/ml dl-polyornithine in water. The primary cultures consisted of 95% astrocytes as judged by immunocytochemical staining of glial fibrillary acidic protein.

Three days before the experiment, the serum-containing medium was removed and cells were transferred to a chemically defined medium consisting of serum-free DMEM:Ham's F12 (1:1, v:v) medium supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite.

2.3. Western blot analysis of PKCζ

After stimulation, cells were washed with ice-cold PBS and subsequently homogenized in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml benzamidine. Soluble and particulate fractions were obtained after centrifugation for 60 min at $40000\times g$. Proteins were then resolved by SDS-PAGE and transferred onto nitrocellulose. Immunodetection of PKCζ was carried out by incubating membranes with anti-PKCζ polyclonal antibody and developing with an enhanced chemiluminescence reaction kit (Amersham).

2.4. Immunocytochemical procedures

Astrocytes were grown in glass coverslips in serum-containing medium and at three weeks made quiescent by serum starvation. Cells were stimulated with different agents, washed with phosphate buffer saline (PBS) and coverslips immediately immersed in 3.7% formaldehyde/PBS for 5 min. After washing in PBS, cells were treated with

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Abbreviations: C_2-ceramide, *N*-acetylsphingosine; C_8-ceramide, *N*-octanoylsphingosine; DHC, dihydro-*N*-acetylsphingosine; IL, interleukin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PC-PLC, phosphatidylcholine phospholipase C; PKC, protein kinase C; SM, sphingomyelin; TNF, tumor necrosis factor

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0.05% Triton X-100 and subsequently incubated with 40 μg/ml polyclonal anti-PKCζ antibody for 60 min and fluorescein-conjugated anti-rabbit IgG for 60 additional min. Coverslips were then mounted with glycerol containing 0.1% *p*-phenylenediamine and subjected to fluorescence microscopy.

2.5. Phosphorylation of PKCζ

Phosphorylation of PKCζ was performed after loading of cells with ^{32}Pi and immunoprecipitation as previously described [17]. Immunoprecipitation was carried out by incubation with 7.5 μg/ml of anti-PKCζ polyclonal antibody and precipitation with agarose-linked protein A. Phosphorylation was determined in the immunoprecipitates by SDS-PAGE and autoradiography. Gels were previously stained with Coomasie blue in order to verify the appropriate loading of the gels. Autoradiography Fuji films were subjected to densitometric analysis using the Sigma-Gel program.

3. Results and discussion

3.1. Translocation of PKCζ and identification by immunoblotting

To study whether SMase and ceramides may have an effect on the subcellular distribution of PKCζ, primary cultures of astrocytes were treated with neutral SMase or cell-permeable ceramide analogs, C_2-ceramide and C_8-ceramide. As shown in Fig. 1, PKCζ appears to be expressed in primary cultures of astrocytes as a single 75 kDa isoform in both the particulate and the soluble fraction, with approximately equal amounts of total enzyme in each fraction in non-stimulated cells (55% and 45% respectively) [18]. C_8- and C_2-ceramide, as well as exogenous SMase, induced a significant increase in immunoreactive PKCζ in the particulate fraction with a concomitant decrease in the soluble fraction (Fig. 1). The time course of C_8-ceramide-induced PKCζ translocation to the particulate fraction is shown in Fig. 2. PKCζ redistribution was already evident after 5 min treatment and was maximal after 15 min of ceramide stimulation. These findings indicate that activation of the SM pathway induces PKCζ translocation to the particulate fraction of rat astrocytes.

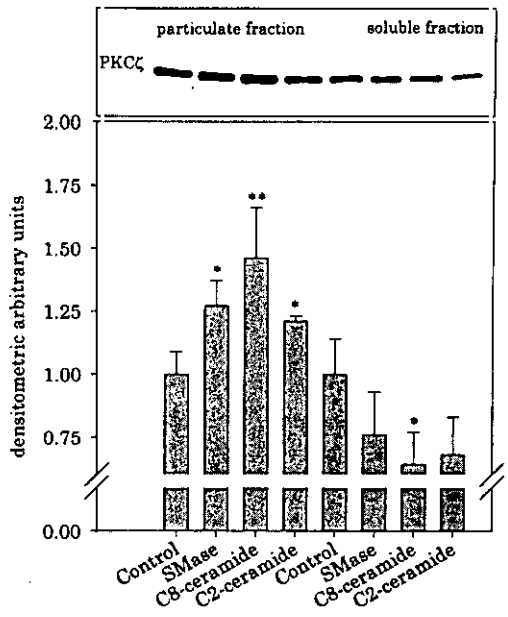

Fig. 1. Localization of PKCζ by immunoblotting. Astrocytes were stimulated with vehicle, 1 U/ml SMase (*S. aureus*), 25 μM C_8-ceramide or 25 μM C_2-ceramide for 15 min. The upper panel shows a representative luminogram of the immunoblots. The lower panel shows the means ± S.D. of densitometric analyses from three independent experiments expressed in arbitrary units referred to densitometric units from control cells. Statistical analysis was performed by Student's *t*-test. Significantly different vs. the corresponding subcellular fraction of control cells: *: $P < 0.025$; **: $P < 0.01$.

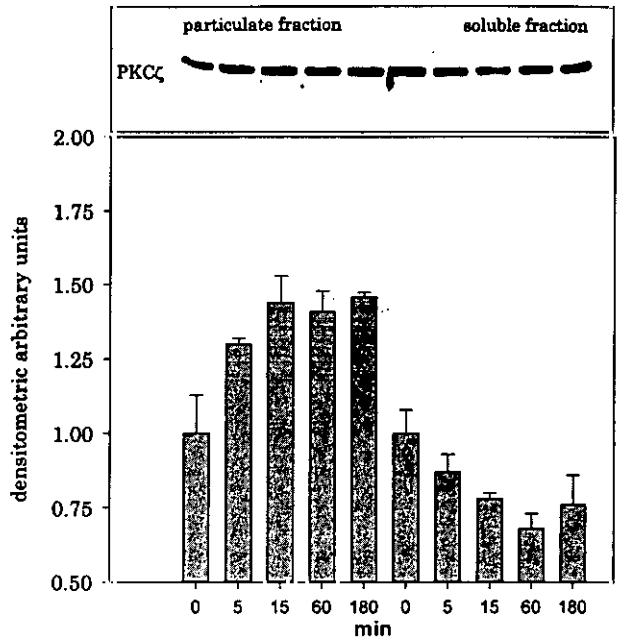

Fig. 2. Time course of C_8-ceramide-induced translocation of PKCζ. Astrocytes were stimulated with 25 μM C_8-ceramide for 0, 5, 15, 60 and 180 min. The upper panel shows a representative luminogram of the immunoblots. The lower panel shows the means ± S.E.M. of densitometric analyses from two independent experiments expressed in arbitrary units referred to densitometric units from control cells.

3.2. Redistribution of PKCζ in primary astrocytes

To further study the intracellular localization of PKCζ, immunocytochemical analyses were performed. As shown in Fig. 3, exposure of cells to either SMase or ceramide analogs induced PKCζ translocation to a perinuclear area. The effect of added SMase indicates that endogenously produced ceramides are also able to induce PKCζ cellular redistribution, therefore corroborating the specificity of ceramide analog action. Perinuclear signal was stronger in SMase-treated and C_8-ceramide-treated cells (Fig. 3C and D) than in C_2-ceramide (Fig. 3E). This may be probably due to the longer alkyl chain of the former, which makes it more similar to natural occurring ceramides. Like in Western blot analysis (see above), C_8-ceramide and SMase-generated ceramides produced a greater effect than the short chain C_2-ceramide. Therefore alkyl chain length may play an important role in ceramide action. This effect has also been observed in raf-1 activation by ceramides [19]. The emerging hypothesis from this observations is that such structural requirements may explain divergent effects of different ceramides as a consequence of its subcellular origin, and the specific SM pool involved [20]. In this context, a different action of intracellularly generated ceramides and exogenously added ceramides in the induction of apoptosis has been recently reported [21].

The specificity of the effect of ceramides is supported by the fact that inactive ceramide analog DHC did not induce any change in the subcellular localization of PKCζ (Fig. 3B).

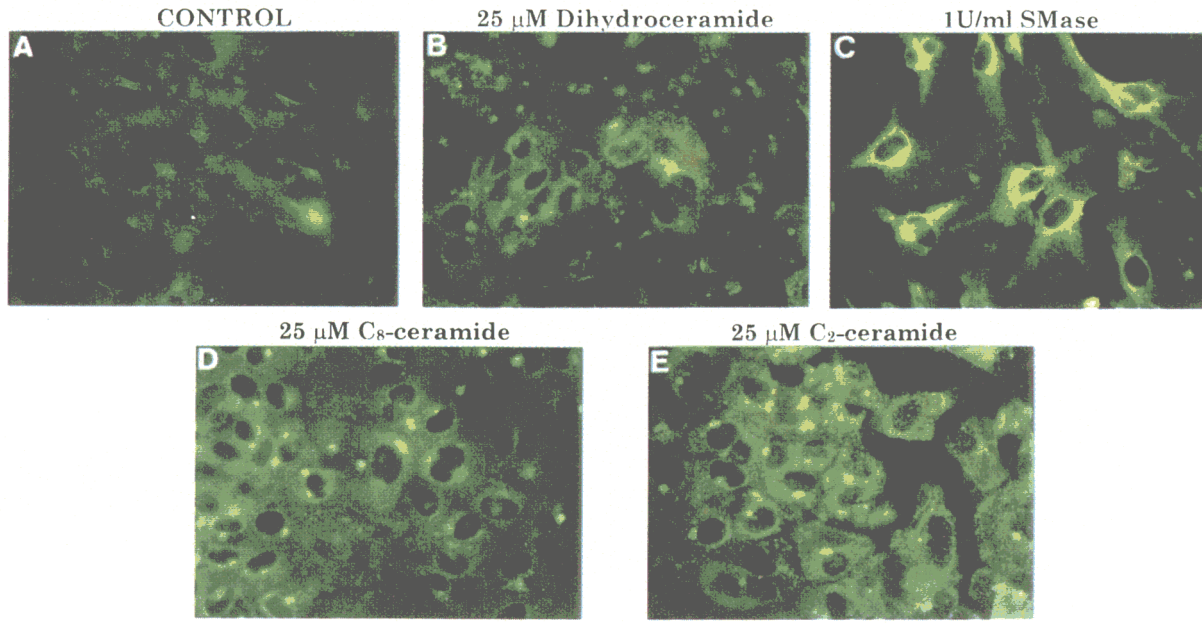

Fig. 3. Immunofluorescence localization of PKCζ in primary astrocytes. Cells were stimulated for 15 min with vehicle (A); 25 μM DHC (B); 1 U/ml SMase (*S. aureus*) (C); 25 μM C_8-ceramide (D) and 25 μM C_2-ceramide (E).

These data are in line with previous observations by other authors who have also found PKCζ located in a perinuclear area in intimate association with the β-tubulin fraction of the cytoskeleton [22,23]. PKCζ has also been found associated to the Golgi apparatus and probably involved in the formation of transport vesicles [24].

3.3. Phosphorylation state of PKCζ

PKC translocation has been related to its functional state [8]. One of the molecular events observed during PKC activation is an increase in its phosphorylation state [25]. To investigate the effect of ceramides in the phosphorylation level of PKCζ, astrocyte cultures were incubated with $^{32}P_i$, and then immunoprecipitation studies were carried out. Fig. 4 shows that short-term treatment with ceramide analogs induced an increase in the phosphorylation state of PKCζ, that was specific for biologically active ceramide analogs, as shown by the lack of effect of DHC. Subcellular redistribution of PKCζ to the particulate cell fraction was in the same interval of time than ceramide-induced increase of PKCζ phosphorylation, indicating that both events may be related.

The precise mechanism of the regulation of PKCζ activity is still controversial. Contradictory results have been reported regarding the subcellular redistribution of PKCζ upon activation. Numerous reports indicate that active PKCζ is found concentrated in membrane fractions or cytoskeleton structures [15,22,24,26]. Translocation of PKCζ from the cytosol to nuclear myofibrilar fraction occurs in the ischemic fetal brain [27]. However, recent data have shown a translocation of activated PKCζ to the cytoplasm, with a concomitant increase in the phosphorylation state and activity of the enzyme in response to changes in intracellular cAMP levels [28]. This indicates that the phosphorylated enzyme would display a diminished membrane affinity [8]. Association of PKCζ with both cytosol and membranes may be mediated through a recently identified protein kinase C-binding protein, i.e. zeta-interacting protein which has been shown to interact with the regulatory domain of the kinase [29].

The importance of ceramide signaling in rat astrocytes has been evidenced in our laboratory by the fact that ceramide analogs, SMase and PC-PLC induce NGF production in these glial cells [16,17]. We report here that both SMase and ceramide analogs induce the translocation of PKCζ to a perinuclear membrane region and increase the PKCζ phosphorylation level, suggesting the possible involvement of PKCζ in ceramide signaling in primary cultures of rat astrocytes.

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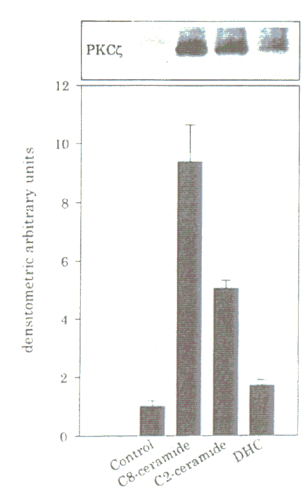

Fig. 4. Phosphorylation of PKCζ. ^{32}P-labelled astrocytes were stimulated with vehicle, 25 μM C_8-ceramide, 25 μM C_2-ceramide, and 25 μM dihydroceramide for 15 min. The upper panel shows a representative autoradiogram. The lower panel shows the means ± S.E.M. of densitometric analyses from two independent experiments expressed in arbitrary units referred to densitometric units from control cells.

assistance in cell culturing. This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAF 96/0113) and Fondo de Inevestigaciones Sanitarias (97/0039-01).

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RESULTADOS Y DISCUSIÓN

El efecto de la PC-PLC en la síntesis y secreción de NGF en cultivos primarios de astrocitos tiene un componente dependiente y otro independiente de la actividad de cPKCs, sensible a Ca^{2+} y DAG. Como aproximación al estudio de uno de los posibles sistemas de transducción de señales independiente de cPKCs, estudiamos uno de los componentes del grupo de las PKCs atípicas, la PKC ζ (Hoffman, 1997). Los niveles de PKC ζ, no disminuyen por exposición prolongada a ésteres de forbol, es decir no experimentan "downregulation", tanto en astrocitos, como en la línea de glioma C6 (Ballestas y Benveniste, 1995; Chen et al., 1995). Los datos obtenidos, muestran como la PC-PLC induce un cambio en la localización subcelular de PKC ζ, acompañado de un aumento del estado de fosforilación de la enzima en la línea de glioma C6. Las variaciones en el estado de fosforilación pueden reflejar cambios en el estado de activación de la isoforma de PKC estudiada (Jaken, 1996). Estas observaciones son compatibles con que la PKC ζ pueda desempeñar un papel en la regulación de la producción de NGF por células gliales inducida por PC-PLC resistente a BIM y "downregulation" de PKC.

La estimulación de la producción de NGF promovida por ceramidas en astrocitos presenta coincidencias con la regulación por la PC-PLC que sugieren que podrían actuar por rutas comunes de señalización celular. En efecto, la estimulación de astrocitos por análogos permeables de ceramida y SMasa exógena afectan a la PKC ζ de manera semejante a lo observado con la PC-PLC. El aumento en los niveles intracelulares de ceramida induce una redistribución de la PKC ζ, que pasa de una localización homogénea en la célula a estar concentrada en una región perinuclear. Paralelamente a la translocación de PKC ζ inducida por las ceramidas, se produce un aumento en los niveles de fosforilación de la enzima. Recientemente, estudios más detallados utilizando microscopía electrónica han permitido observar que en la activación de células PC12 se induce la translocación de PKC ζ al núcleo, donde es capaz de unirse a la cromatina (Wooten et al., 1997). Estas observaciones, junto con el papel de la PKC ζ en la activación de NF-κB por la SMasa (Lozano et al., 1994), apuntan un posible papel de la PKC ζ en los efectos de regulación de la expresión génica inducidos por los sistemas de transducción de señales que involucran a esta quinasa.

3. REGULACIÓN DE LA SÍNTESIS Y SECRECIÓN DE NGF POR TNFα Y LPS EN ASTROCITOS EN CULTIVO PRIMARIO

Este apartado se refiere al estudio de la regulación de la producción de NGF por la citoquina pro-inflamatoria TNFα y el efecto del lipopolisacárido bacteriano (*Escherichia coli).* Algunos de los efectos biológicos de las citoquinas proinflamatorias, como el TNFα y la IL-1β están mediados por la vía de transducción de la SMasa y consiguiente generación de ceramidas (Hannun, 1996). Por otro lado TNFα e IL-1β son potentes inductores de la síntesis de NGF (Carman-Krzan et al., 1991; Hattori et al., 1993; Pshenichkin et al., 1994). Por ello resulta de gran interés estudiar la posible implicación de la ruta de las ceramidas en la inducción de la síntesis de NGF por el TNFα, uno de los principales reguladores fisiológicos del proceso de gliosis (Hurwizt et al., 1995; Merril y Benveniste, 1996). La segunda parte del capítulo aborda el efecto inductor en la síntesis de NGF de la exposición al LPS de los astrocitos en cultivo primario.

Por último, se estudia el papel regulador del factor nuclear de transcripción NF-κB en la expresión del gen de NGF. Este factor de transcripción está relacionado con algunas de las respuestas celulares inducidas por TNFα y LPS, así como con la vía de generación de ceramidas (Yang et al., 1993; Machleidt et al., 1994; Wiegman et al., 1994; Kohler y Joly, 1997) y podría por tanto explicar su efecto activador de la síntesis de NGF.

Evidence for the lack of involvement of sphingomyelin hydrolysis in the tumor necrosis factor-induced secretion of NGF in primary astrocyte cultures

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The signal mechanism underlying tumor necrosis factor α (TNFα) upregulation of nerve growth factor (NGF) production was studied in primary rat astrocytes. Since ceramide is also able to induce NGF secretion and because TNFα is a known agonist of the sphingomyelin-ceramide pathway, we investigated whether the TNFα-induced NGF secretion by primary astrocytes is mediated by ceramide. TNFα stimulation of NGF secretion was shown to be independent of protein kinase C, abrogated by the tyrosine phosphoprotein phosphatase inhibitor phenylarsine oxide (PAO), and independent of the activation of the mitogen activated protein kinase cascade. In marked contrast, inhibition of MAP kinase counteracted the NGF secretion induced by ceramide. TNFα stimulation of the nuclear transcription factor NF-κB was prevented by cell pretreatment with PAO, whereas ceramide and sphingomyelinase had a marginal effect on NF-κB activation. Moreover, TNFα failed to activate the sphingomyelin pathway, as indicated by the lack of SPM degradation and the absence of ceramide generation. To further clarify the role of NF-κB in NGF synthesis, EMSA were performed with a NF-κB site from the NGF promoter. The absence of significant binding of NF-κB to the NGF gene promoter indicates the existence of an indirect mechanism for NF-κB regulation of NGF synthesis. Altogether, our data strongly suggest that TNFα-mediated upregulation of NGF occurs independently of ceramide generation.

Key words: astrocytes, ceramide, Nerve growth factor, NF-κB, sphingomyelin, tumor necrosis factor.
Abbreviations used: BIM, bisindolylmaleimide GF109203X; DAG, diacylglycerol; db-cAMP, dibutyryl cyclic AMP; EMSA, electrophoretic mobility shift assay; FK, forskolin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PAO, phenylarsine oxide; PC, phosphatidylcholine; PKC, protein kinase C; SPM, sphingomyelin; TNF, tumor necrosis factor

1. Introduction

Pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) are important mediators of inflammation and immune responses in the CNS. Such responses are followed by the rapid activation of brain resident macrophages and subsequent activation of astrocytes (Merrill and Benveniste, 1996). One of the responses of reactive astrocytes is the synthesis and release of neurotrophic factors such as nerve growth factor (NGF) (Brodie, 1996; Yu et al., 1996). NGF production by glial cells during such circumstances may limit the extent of neuronal loss and promote regenerative processes to restore neuronal function (Pshenichkin et al., 1994).

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NGF synthesis by astrocytes is a finely regulated process which includes complex interactions among different signaling pathways. Pro-inflammatory cytokines such as TNFα are well known stimulators of NGF synthesis (Hattori et al., 1993; Pshenichkin et al., 1994). TNFα is a pleiotropic cytokine which exerts most of its biological effects, including NGF induction, through the 55 kDa TNFα receptor (Hattori et al., 1996), which is the TNFα receptor predominantly expressed in astrocytes (Dopp et al., 1997). Some of the early events triggered by TNFα include the activation of phospholipase A_2 (Jayadev et al., 1994), neutral and acidic sphingomyelinases (SPMases) as well as phosphatidylcholine-phospholipase C (PC-PLC) (Schütze et al., 1992; Andrieu et al., 1995; Wiegman et al., 1994). The signaling cascade involving sphingomyelin (SPM) hydrolysis and subsequent ceramide generation constitutes the so-called sphingomyelin-ceramide pathway (Hannun, 1996; Spiegel et al., 1996).

We have recently demonstrated that N-acetylsphingosine (C_2-ceramide), a cell-permeant ceramide analog, and exogenous bacterial SPMase, which elevates intracellular ceramide, are potent inducers of NGF synthesis by primary astrocytes (Galve-Roperh et al., 1997a). Ceramide action as second messenger may involve stimulation of different molecular targets as ceramide-activated protein kinase, ceramide-activated protein phosphatase, and atypical PKC ζ (Hannun, 1996; Spiegel et al., 1996). As a matter of fact, ceramides are able to trigger PKC ζ translocation and phosphorylation in primary astrocyte cultures (Galve-Roperh et al., 1997c). Activation of the MAPK cascade by the stimulation of SPM-ceramide pathway has also been reported, and may be related to Raf-1 activation (Huwiler et al., 1996), which may in turn be activated by direct interaction with PKC ζ (Van Dijk et al., 1997).

Another characteristic intracellular action induced by TNFα is the early activation of the nuclear transcription factor NF-κB (Schütze et al., 1992). The exact role of ceramide generation in TNFα-activation of NF-κB is still controversial (Hannun, 1996). Thus, some reports indicate that ceramide generation through acidic SPMase may be responsible for NF-κB activation (Machleidt et al., 1994; Wiegman et al., 1994), whereas others have not found evidence for the role of ceramide in NF-κB stimulation (Betts et al., 1993; Andrieu et al., 1995; Gamard et al., 1997; Zumbansen and Stoffel, 1997).

This work was therefore undertaken in order to further study the role of ceramide and other signal transduction pathways involved in TNFα-mediated NGF synthesis and secretion by primary astrocytes. The role of nuclear transcription factor NF-κB in NGF gene regulation was also assessed.

2. Materials and methods

2.1 *Materials*

Tissue culture plastic wares were from Nunc (Denmark). Culture media and fetal calf serum were from Biowhitakker (Belgium). The bisindolylmaleimide GF 109203X, forskolin, PD 098059 and cell permeable ceramide analogs were from Calbiochem (USA). TNFα was from PreproTech-Tebu (France). T4 polynucleotide kinase, NF-κB protein and NF-κB consensus oligonucleotide were from Promega (France). Bacterial SPMase (*S. aureus*), dibutyryl cyclic AMP and the rest of reagents were from Sigma Chemicals (USA).

2.2 *Primary cultures of astrocyte cells*

Cortical astrocytes were derived from 1-2 day old rats and cultured as previously described (Galve-Roperh et al., 1997a). Cells were seeded at a density of $3x10^4$ cells/cm^2 on plastic plates previously coated with 5 μg/ml dl-polyornithine in water. Cells were cultured for 3 weeks in basal medium consisting of a mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 (1:1, v/v), with 0.66% glucose, 5 μg/ml streptomycin, 5 U/ml penicillin, and supplemented with 10% fetal calf serum. The primary cultures consisted of 95% astrocytes as judged by immunocytochemical staining of glial fibrillary acidic protein. Primary cultures of astrocytes were incubated for three days before the experiments, in chemically-defined medium (consisting

of serum-free basal medium supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite).

2.3 ELISA of secreted NGF

For extracellular NGF determination, cell supernatants were collected 48 h after treatment as described in the text, diluted in one volume of phosphate buffer saline (PBS) containing 0.1% Tween 20 and 0.5% gelatin, and conserved frozen at -20 °C until quantitation. NGF released by the cells was assayed in triplicate, by a double-site ELISA, using a monoclonal anti-NGF antibody, coupled or not to β-galactosidase according to an experimental protocol described before (Laviada et al., 1995).

2.4 Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were obtained according to a previously described method (Andrieu et al., 1995). Briefly, cell pellets were homogenized at 4 °C in 400 μl of buffer A (10 mM Hepes, pH 7.8, 10 mM KCl, 1mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin). The cells were allowed to swell for 15 min on ice, Igepal CA-630 (0.6% final concentration) was added, and the tubes were vortexed. Nuclei were pelleted by centrifugation at 1200 x g for 20 min at 4 °C. The supernatant containing the cytosolic fraction was centrifuged at 14 000 x g and the resulting supernatant was used for Western blot analysis. The nuclei were suspended in buffer A and after sedimentation at 2000 x g for 20 min, they were suspended in 25 μl of buffer C (20 mM Hepes, pH 7.8, 400 mM NaCl, 1mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) and incubated for 30 min on ice. After centrifugation at 15 000 x g for 20 min, the supernatant was collected and stored at -80 °C. Protein concentrations were determined by the bicinchoninic acid method.

2.5 Electrophoretic mobility shift assays

Oligonucleotide probes used were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP according to the supplier's instructions. Binding reactions were performed in a total volume of 20 μl with the radiolabeled oligonucleotide (250000 cpm), binding buffer (containing 2 mM Hepes, pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiotreitol, 4% glycerol, 1.2 μg poly (dIdC), and 0.4 μg of bovine serum albumin) and 5 μg of nuclear extract. After incubation for 20 min at room temperature, the samples were subjected to electrophoresis on 4% acrylamide gel for 3-4 h at 100 V. Dried gel was exposed to an X-ray film (Hyperfilm, Amersham) at -80° C or quantified by PhosphorImager 445 SI (Molecular Dynamics).

The following oligonucleotides were used in EMSA: NF-κB consensus, AGT TGA GGG GAC TTT CCC AGG C; mutated NF-κB, AGT TGA TTC TCG AAA CCC AGG C; NF-κB$_{NGF}$, GCA TTG CAG GGG CCT CCC AGG AG and mutated NF-κB$_{NGF}$, GCA TTG CAT AGC CCT CCC AGG AG.

2.6 Western blot analysis of IkB-α

Cytosolic extracts were subjected to SDS-PAGE on 8% polyacrylamide gels (Galve-Roperh et al., 1997c). Then proteins were transferred onto Hybond-ECL membrane (Amersham, U.K.). Blots were blocked with 5% fat-free dried milk, and subsequently incubated with IkB-α polyclonal antibody (Santa Cruz, USA). Membranes were then incubated with secondary anti-rabbit peroxidase-labeled antibody and finally subjected to luminography with an ECL detection kit (Amersham, U.K.).

2.7 Metabolic labeling of cellular choline-phospholipids and lipid analysis

Primary cultures of astrocytes were incubated before the experiment in the chemically-defined medium with 1 μCi/ml of [methyl-^{3}H]choline. After 72 h incubation, the radioactive medium was removed and cells were chased for 2 h in the chemically-defined medium. Then, cells were stimulated with TNFα for the indicated times, scraped and inmediately subjected to low speed centrifugation and frozen at -20 °C.

Lipid extraction and analysis were performed according to previously described procedures (Andrieu et al., 1994). Briefly, cell pellets were suspended in distilled water and cells were disrupted at 4°C by brief sonication (10 s). An aliquot was taken for protein determination. The remaining was extracted with 2.5 ml of chloroform/methanol (2:1, v/v). The lower phase was washed with a synthetic upper phase consisting of chloroform/methanol/water (3:48:47, v/v/v) and evaporated under nitrogen. Then the lipid extract was subjected to mild alkaline hydrolysis as follows: the residue was dissolved in 0.25 ml chloroform and 0.25 ml methanolic 0.5 M NaOH, and incubated overnight at 37 °C. After addition of 0.85 ml of chloroform, 0.25 ml of methanolic 0.5 M HCl, 0.43 ml of water and 0.5 ml of chloroform/methanol (2:1, v/v) the upper phase (containing the [^{3}H]-choline label released from phosphatidylcholine) was counted for radioactivity. The lower phase was washed twice with the synthetic upper phase, and the washings were counted for radioactivity. The organic phase (containing the SPM) was evaporated and counted for radioactivity. In some experiments, the mass of total phospholipids and SPM was quantitated by measuring their phosphate content (Ames, 1966).

Ceramide quantitation was carried out using *E. coli* diacylglycerol kinase (Amersham, UK) and [γ-^{32}P]ATP according to previously published procedures (Van Veldhoven et al., 1992).

3. Results

3.1 TNFα-induced NGF secretion in primary astrocytes is independent of PKC and MAPK activities

In order to investigate the regulation of NGF synthesis by TNFα, primary astrocytes were exposed to the cytokine and the NGF secreted into the extracellular medium was quantified by ELISA. Incubation of astrocytes with TNFα induced a dose-dependent increase in the NGF present in the cell supernatant, with a maximal effect at 50 ng/ml (data not included). The enhanced synthesis and secretion reached a maximal level after 48 h of cytokine stimulation.

In an attempt to clarify the possible signaling pathways involved in TNFα upregulation of NGF, cells were pretreated with the bisindolylmaleimide GF109203X (BIM), a highly specific inhibitor of PKC. We have previously demonstrated that BIM treatment of astrocytes is able to mimic the effect of PKC down-regulation on the regulation of NGF secretion in glial cells (Laviada et al., 1995). BIM did not affect TNFα-stimulated NGF secretion, therefore indicating a PKC-independent mechanism of cytokine effect (Fig. 1A). On the other hand, the cAMP analog dibutyryl-cyclic AMP (db-cAMP) and the cAMP raising agent forskolin (FK) partially inhibited cytokine stimulated NGF secretion (30% and 25% inhibition, respectively).

The MAP kinase dependency of TNFα-induced NGF secretion was studied by pretreatment of the cells with the MEK inhibitor PD098059 (Alessi et al., 1995), which is able to prevent MAP kinase activation in primary astrocytes (Galve-Roperh et al., 1997a). Specific MEK inhibition did not result in a decrease, but rather led to a slight increase, of TNFα-induced NGF secretion, indicating a MAP kinase independent mechanism. This was in marked contrast with NGF induction by cell-permeant ceramide and exogenously added SPMase (Fig. 1B), where incubation with PD098059 resulted in a 80 and 90% inhibition respectively, strongly suggesting that TNFα and ceramides act through different pathways.

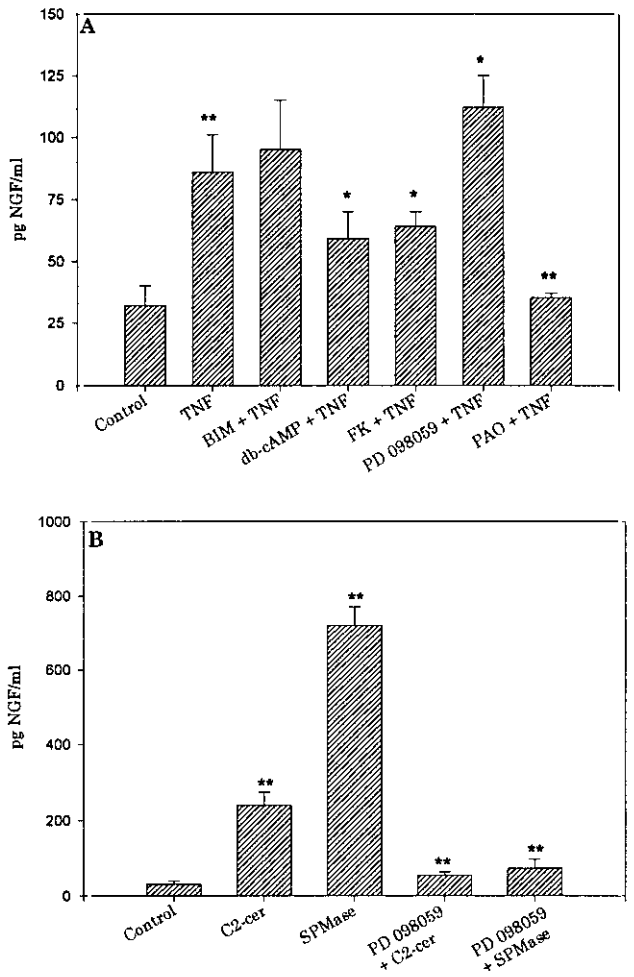

Fig. 1. Regulation of TNFα-, C2-ceramide- and SPMase-stimulated NGF secretion. Primary cultures of rat astrocytes were incubated in the presence of 2 μM BIM, 500 μM db-cAMP, 10 μM FK, 100 μM PD 098059 or 500 nM PAO 1 h prior to 50 ng/ml TNFα (**A**) or 25 μM C2-ceramide, 1 U/ml bacterial SPMase (**B**) treatment for 48 h. NGF in the supernatants was assayed with a double site ELISA. Data are means ± S.E.M. of 3 to 4 independent experiments with determinations in triplicate. Agents alone, which had no effect on NGF secretion have been omitted for clarification. Statistical analysis was performed by Student's t-test; significant differences are indicated: * $P<0.025$, ** $P<0.001$. Statistical analysis for TNFα and ceramide stimulation is related to control cells, and when inhibitors are used the statistics is referred to the values with the stimulant alone.

Preincubation of primary astrocytes with the tyrosine phosphoprotein phosphatase inhibitor phenylarsine oxide (PAO) completely abolished the TNFα-stimulated NGF secretion. PAO has been shown to prevent NF-κB activation by TNFα in both myeloid and epithelial cells (Singh and Aggarwal, 1995). Therefore, these results indicate that TNFα regulation of NGF synthesis is independent of PKC and MAPK activities, is partially inhibited by cAMP raising agents, and may involve the nuclear transcription factor NF-κB.

3.2 Regulation of the nuclear transcription factor NF-κB by TNFα

To determine the effect of TNFα on the nuclear transcription factor NF-κB, electrophoretic mobility shift assays (EMSA) were undertaken. Treatment of astrocytes with TNFα led to the activation of NF-κB, as demonstrated in the EMSA by the formation of a specific band which was competed off by an excess of unlabeled probe. Fig. 2 shows the time course of TNFα-induced NF-κB activation. Nuclear translocation of NF-κB was already evident 5 min after cell stimulation with a maximal effect at 30 min of treatment. Although the nuclear transcription factor AP-1 was also activated in primary astrocytes by TNFα, AP-1 activation was transient, with maximal activation only 5 min after TNFα stimulation and returning to basal levels within 15 min (data not shown).

We further investigated NF-κB regulation by TNFα to identify possible analogies with TNFα-stimulated NGF secretion. As shown in Fig. 3, PAO completely blocked NF-κB activation, in agreement with previous reports indicating the role of tyrosine phosphoprotein phosphatase in NF-κB activation by TNFα (Singh and Aggarwal, 1995). NF-κB activation was slightly inhibited by PKC inhibition as shown by the effect of BIM on TNFα stimulation. Increased intracellular levels of cAMP evoked by FK partially blocked the ability of TNFα to activate NF-κB. Similar results were obtained with db-cAMP. The specifity of NF-κB

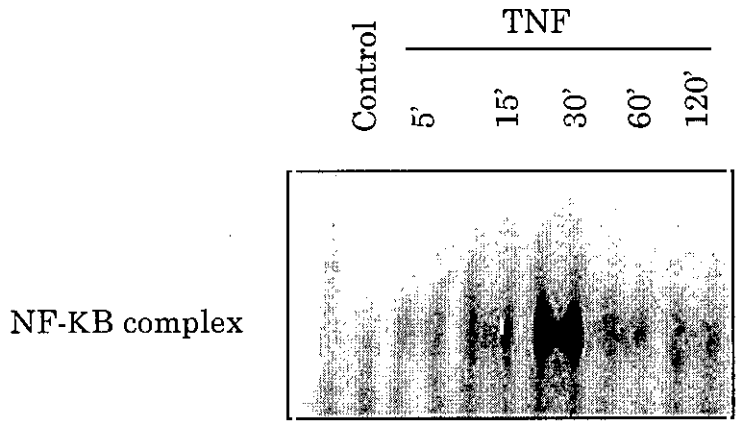

Fig. 2. Time-course of TNFα activation of NF-κB. Primary astrocytes were stimulated for the indicated times with 35 ng/ml TNFα, and then nuclear extracts subjected to EMSA. The gel is representative of two independent experiments.

activation by TNFα was demonstrated by the inhibitory effect exerted by the serine protease inhibitor dichloroisocoumarin (DCIC) (data not shown). Indeed, pretreatment of cells with this protease inhibitor abolished TNFα-induced NF-κB activation, which is concordance with a previous report that demonstrated the existence of a serine-like protease crucial for the control of NF-κB activation by TNFα (Machleidt et al., 1994).

Some of the intracellular effects of TNFα have been related to its ability to induce ceramide generation upon SPM hydrolysis (Hannun et al., 1996; Spiegel et al., 1996). In order to test the possible role of ceramide generation in TNFα stimulation of NF-κB activation, we

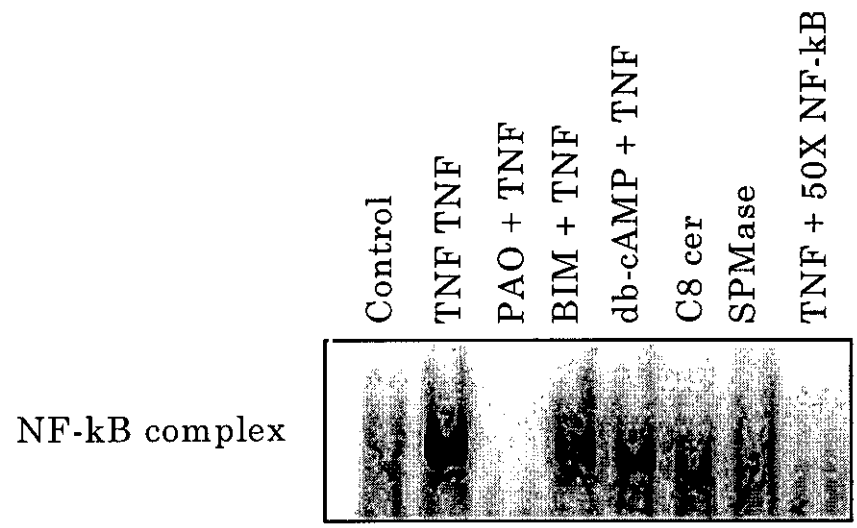

Fig. 3. Regulation of NF-κB activation by TNFα and ceramide. Primary astrocytes were treated for 30 min with 35 ng/ml TNFα, 25 μM C8-ceramide and 1 U/ml SPMase. Where indicated, cells were preincubated for 30 min with 5 μM PAO, 2 μM BIM or 500 μM db-cAMP. Results shown are representative of 3 to 4 independent experiments.

assessed the ability of ceramide treatment to activate NF-κB in primary rat astrocytes. As shown in Fig. 3, treatment with the cell-permeant N-octanoylceramide (C_8-ceramide) or with bacterial SPMase, which elevates the cellular ceramide concentration, also stimulated the nuclear translocation of NF-κB. However, in agreement with previous observations on other cell types (Andrieu et al., 1995), their stimulatory effect was much weaker than that exerted by TNFα.

3.3 IkB-α degradation occurs in concert with NF-κB activation in TNFα-stimulated astrocytes

Dissociation of the inhibitory moiety, IkB, of cytosolic NF-κB complexes allows NF-κB activation and translocation to the nucleus (O'Neill and Kaltschmidt, 1997). Therefore, we measured the levels of IkB-α protein in the cytosol of TNFα- and ceramide-stimulated cells by Western blot. Fig. 4A shows that there is a dramatic depletion of the inhibitory subunit lvels in TNFα-stimulated cells, which occurs in parallel to the observed activation of NF-κB. In agreement with results obtained by EMSA, exogenous SPMase, C_8-, and C_2-ceramides produced a slight decrease in cytosolic levels of IkB-α (Fig. 4A); however, this decrease was not as pronounced as in TNFα-treated cells. Degradation of IkB-α was prevented by cell pretreatment with PAO prior to TNFα or C_8-ceramide stimulation (Fig. 4B).

3.4 The sphingomyelin-ceramide pathway is not activated by TNFα in primary astrocytes

To further investigate whether the SPM-ceramide pathway was activated by TNFα in primary astrocytes, SPM and ceramide levels were measured as described in Materials and Methods. Although slight variations could be observed, TNFα stimulations for up to 120 min did not evoke any significant decrease of SPM levels nor any increase of intracellular ceramide levels (Fig. 5A). SPM mass measurements were carried out leading to identical results (n=3;

data not included). Furthermore, neither SPM nor ceramide levels were affected by TNFα in the C6 glioma cell line (n=3, data not included). Measurement of phosphatidylcholine (PC) and diacylglycerol (DAG) levels also indicated the absence of a pool of PC sensitive to TNFα in astrocytes (Fig 5B).

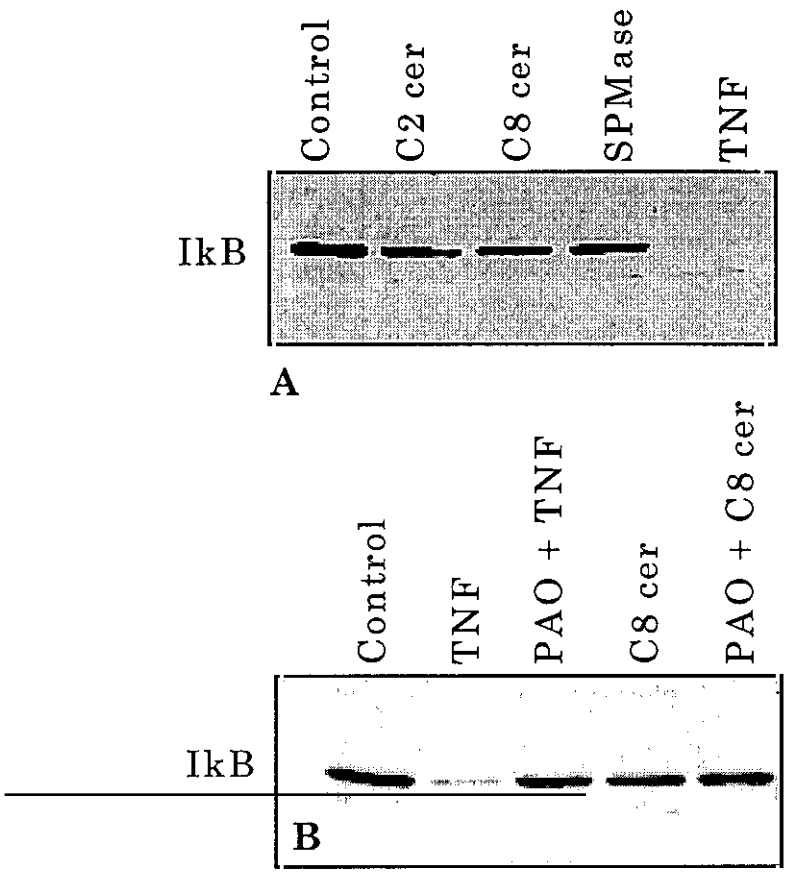

Fig. 4. Western blot analysis of IkB-α subunit on cytosolic extracts from stimulated astrocytes. A/ Cells were stimulated with vehicle, 25 μM C_2-ceramide, 25 μM C_8-ceramide, 1 U/ml SPMase, or 35 ng/ml TNFα for 30 min. B/ Cells were incubated with or without 5 μM PAO for 30 min prior to TNFα or C_8-ceramide stimulation.

3.5 NF-κB does not directly regulate NGF gene expression

The NGF gene promoter contains a NF-κB like binding site located 669 base pairs upstream of the TATA signal (Jehan et al., 1993). To examine whether this site is a potential target for activated NF-κB to regulate NGF gene transcription, gel shift assays were performed with purified nuclear transcription NF-κB and with the oligonucleotides of the proposed site of regulation. Fig. 6 shows that, although this NF-κB$_{NGF}$ probe is able to bind specifically the nuclear transcription factor (see result with mutated oligonucleotide), the intensity of the binding is weak as compared to the binding to NF-κB consensus

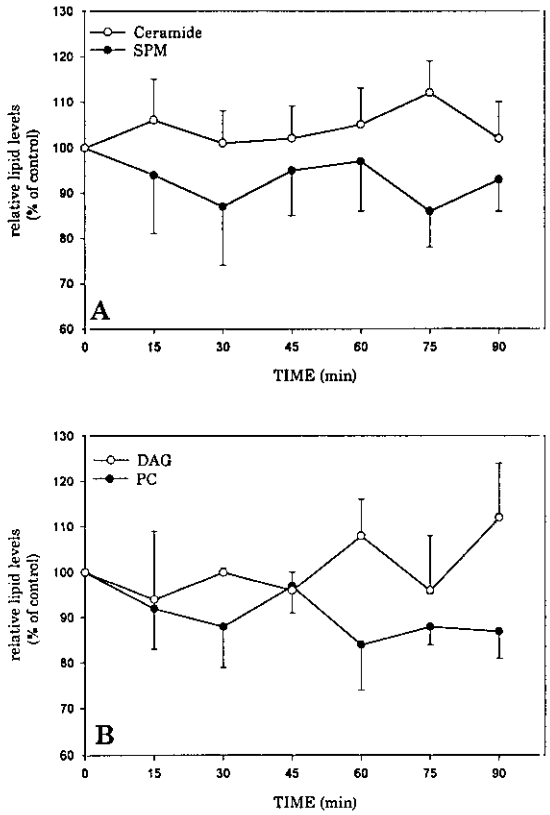

Fig. 5. Lipid quantification after TNFα stimulation. Primary rat astrocytes were labeled with [^{3}H]choline for 72 h. Then, cells were incubated with 35 ng/ml TNFα and at the indicated time points incubations were stopped. SPM (Fig 5A, -●-) and PC (Fig. 5B, -●-) levels were determined as described in "Materials and Methods". Phospholipid levels are expressed as percentage of the value observed at time 0. Values represent the mean ± S.E.M. of 4 different experiments. Ceramide (Fig. 5A, -○-) and DAG (Fig. 5B, -○-) levels were determined by the DAG kinase method. Values are expressed as percentage of radiactivity at time 0 of 3 independent experiments. Statistical analysis using the Student's t-test revealed no significant differences.

oligonucleotide. In fact, cellular NF-κB present in the nuclear extracts from TNFα-stimulated cells was not able to bind to the NF-κB$_{NGF}$ site (data not shown).

4. Discussion

Results from the present report point to a differential effect of TNFα versus exogenous ceramide or SPMase in the regulation of NGF synthesis and secretion by primary astrocytes. In spite of the many efforts to elucidate the mechanisms that control NGF synthesis, the regulation of this complex process remains largely unknown. We have previously described the involvement of exogenous PC-PLC in the regulation of NGF synthesis by astrocytes (Laviada et al., 1995). PC-PLC in turn has also been reported to mediate TNFα activation of NF-κB through acidic SPM breakdown (Schütze et al., 1992; Wiegmann et al., 1994). NGF induction by PC-PLC is partially dependent on PKC activity (Laviada et al., 1995), whereas TNFα induction of NGF synthesis in primary astrocytes seems to be independent of PKC activity (the present report). This is also true for NGF induction by cell-permeant ceramides and exogenous bacterial SPMase (Galve-Roperh et al., 1997).

The existence of crosstalks among different signaling pathways, which allows a tight control of NGF synthesis, is evidenced by the inhibitory effect of increased intracellular cAMP levels on TNFα-stimulated NGF production. We have previously described a similar inhibitory effect of the cAMP cascade on ceramide- and lipopolysaccharide-induced NGF synthesis (Galve-Roperh et al., 1997a; 1997b), although the exact mechanism underlying this process is not well understood. Increased cAMP levels have been shown to prevent MAP kinase activation in astrocytes (Kurino et al., 1996; Willis and Nisen, 1996). However, although TNFα increases p42/p44 MAP kinase activity in primary astrocytes (unpublished observation), this activation would not be responsible for the induction of NGF synthesis, as shown by the lack of inhibitory effect of PD098059 (Fig. 1). Our data rather indicate that NGF induction by TNFα in primary astrocytes is independent of MAP kinase activity, in sharp contrast with the effect of ceramides, which activate NGF synthesis by a p42/44 MAPK activity-dependent mechanism (Galve-Roperh et al., 1997a, and present report). The inhibitory effect of cAMP may also be attributed to the observed decrease of TNFα-activation of NF-κB (the present report; Pahan et al., 1997).

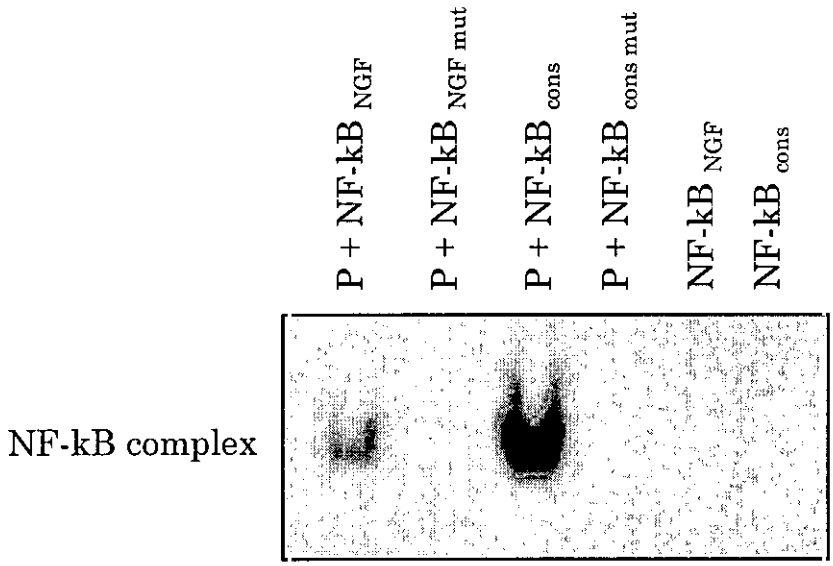

Fig. 6. Analysis of NF-κB binding to different oligonucleotides. Gel retardation assays were performed with 400 ng of the purified NF-κB protein (P) and the corresponding radiolabeled probes. Lanes 1, P + NF-κB NGF probe; 2, P + NF-κB NGF mutated probe; 3, P + NF-κB consensus probe; 4, P + NF-κB consensus mutated probe; 5, free NF-κB NGF probe and 6; free NF-κB consensus probe.

Our results show that in primary rat astrocytes the nuclear transcription factor NF-κB is rapidly activated by TNFα stimulation. The ability of TNFα to activate NF-κB is well established, but much controversy still exists about the signaling mechanisms involved in such a stimulation. Some reports indicate that ceramide generation, by acidic SPMase, may be responsible for NF-κB activation (Schütze et al., 1992; Machleidt et al., 1994; Wiegmann et al., 1994). In contrast, others have not found evidence for the involvement of ceramide in NF-κB stimulation (Betts et al., 1993; Andrieu et al., 1995; Gamard et al., 1997). Moreover, evidence has been presented against a role of acidic SPMase in the effects of TNFα (Andrieu et al., 1994; Kuno et al., 1994; Zumbansen and Stoffel, 1997). In our hands, exogenous SPMase and C_8-

ceramide, which stimulate NGF synthesis to a much greater extent than TNFα, activated NF-κB in primary astrocytes, although to a lesser extent than TNFα. In addition, IkB-α egradation was less affected by ceramides than by TNFα. Recently, Modur et al. (1996) have described that in endothelial cells TNFα is able to activate Raf-1 through a ceramide-dependent pathway, whereas NF-κB activation is achieved by a ceramide-independent mechanism. Our data suggests that primary astrocytes may behave in such a way. As a matter of fact, ceramide is able to induce subcellular redistribution of the atypical PKC ζ in glial cells (Galve-Roperh et al., 1997c), and this effect coincides with increased Raf-1 phosphorylation and MAPK activity (Galve-Roperh et al., 1997a).

The existence of a ceramide-independent mechanism for the TNFα-induced stimulation of NF-κB was confirmed by the lack of significant SPM hydrolysis upon cell stimulation. It is worth noting that the absence of activation of the SPM-ceramide pathway by TNFα might be a common feature of glial cells, since murine C6 glioma cells and human U251MG glioma cells are also resistant to TNFα-stimulated SPM hydrolysis (data not shown; Richard et al., 1996). Moreover, although ceramides are able to stimulate stress-activated protein kinase in glial cells, TNFα activation of SAPK does not seem to involve the ceramide pathway (Zhang et al., 1996). The TNFα-sensitive SPM pool involved in signal transduction has been suggested to be located in the inner leaflet of the plasma membrane (Andrieu et al., 1996). The absence of significant variations of SPM mass levels, together with the absence of ceramide generation, rule out the possibility that metabolic labeling with [^{3}H]choline may not be able to label the TNFα-sensitive signaling pool of SPM in confluent astrocytes.

The origin of the endogenous ceramides responsible for the induction of NGF synthesis in the cell may be linked to other possible generation sources of ceramide than SPM hydrolysis, e.g. resulting from stimulated "*de novo*" synthesis or inhibited degradation. These processes have already been shown to mediate some of the biological effects of sphingolipids (Bose et al., 1995; Bielawska et al., 1996; Paumen et al., 1997). For instance, the generation of ceramide by a mechanism involving a reduced rate of catabolism would lead to a progressive and slow accumulation of the lipid within the cell, and this may be of great importance in regulating long-term responses (Bielawska et al., 1996).

In summary, our results are in line with previous reports pointing to the existence of ceramide-dependent and ceramide-independent effects of TNFα. Our data provide strong evidence that TNFα and ceramide act by different mechanisms in the regulation of NGF secretion by astrocytes. This is evidenced by their different dependency on MAP kinase activity, the lack of SPM-ceramide activation by TNFα, and their different ability to activate NF-κB. Finally, our results indicate that, although NF-κB transcription factor does not appear to play a direct role on NGF gene transcription, TNFα-induction of NGF synthesis may rely on previous NF-kB activation.

Acknowledgments

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Regulation of Nerve Growth Factor Secretion and mRNA Expression by Bacterial Lipopolysaccharide in Primary Cultures of Rat Astrocytes

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The present work was undertaken to study the effect of bacterial lipopolysaccharide (LPS), a potent activator of the host inflammatory response, on the synthesis of nerve growth factor (NGF) by newborn rat brain astrocytes. Treatment of primary rat astroglial cells cultured in chemically defined medium with LPS resulted in a dose-dependent accumulation of NGF mRNA, and an increased release of NGF protein in the cell medium. NGF mRNA levels were maximal after 24 hr of stimulation (8-fold increase), whereas extracellular NGF peaked after 72 hours of treatment (17-fold increase). This dramatic increase of extracellular NGF was abrogated if cells were treated with actinomycin D or cycloheximide, a fact which implies that the accumulation of extracellular NGF by LPS-treated cells requires DNA transcription and RNA translation. Stimulation of NGF synthesis and secretion was: (i) unaffected by treatment with the protein kinase C inhibitor bisindolylmaleimide, and (ii) prevented by forskolin and 3-isobutyl-1-methylxanthine, two agents which increase cAMP levels. Inhibition of LPS effect was also obtained with apigenin, a proposed inhibitor of the mitogen-activated protein kinase pathway. Results thus show that LPS stimulates NGF synthesis by astroglial cells through a mechanism that is independent of protein kinase C (PKC), antagonized by cAMP-elevating agents, and probably mediated by the mitogen-activated protein kinase cascade. The data raise the possibility that LPS exerts stimulatory effects on NGF synthesis that are independent of those elicited by astrocyte-derived inflammatory lymphokines such as IL-1β, TNFα or TGFβ1. J. Neurosci. Res. 49:569–575, 1997. © 1997 Wiley-Liss, Inc.

Key words: lipopolysaccharide; nerve growth factor; astrocytes; protein kinase C

INTRODUCTION

Bacterial lipopolysaccharide (LPS) is the sole lipid present in the outer membrane of Gram-negative bacteria. It acts as a potent activator of the host inflammatory response. In the central nervous system (CNS), local injection of LPS is followed by a rapid activation of brain resident macrophages, the microglial cells, and by a recruitment of peripheral monocytes or macrophages (Montero-Menei et al., 1996). However, astrocytes become activated in a second phase of the inflammatory process. It is presently well established that astrocytes constitute an important auxilliary cell controlling the immunological status of the CNS. These cells, when activated with LPS, produce lymphokines or cytokines such as nitric oxide, IL-1β, IL-6, TGF-β1 and TNFα (Kimberlin et al., 1995; Schmidlin and Wiesinger, 1995; Willis and Nisen, 1995; Slegers and Joniau, 1996) which are actively driving the immune reactions. However, astrocytes are also a source of neurotrophic factors, and

Abbreviations: BIM, bisindolylmaleimide; cAMP, cyclic AMP; FCS, fetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; PKC, protein kinase C; PMA, phorbol 12β-myristate 13α-acetate; TGFβ1, transforming growth factor β1; TNFα, tumor necrosis factor α; Tyr, tyrphostin.

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these different mediators, when applied exogenously alone or in combination, can act as inducers of the synthesis of the neurotrophic factor nerve growth factor (NGF), at least in vitro, and possibly in vivo (Pshenichkin et al., 1994; Plüss et al., 1995; Hattori et al., 1996). In a more general way, a relationship between CNS inflammation and NGF accumulation exists, since levels of this factor appear significantly increased in cerebrospinal fluids of multiple sclerosis patients (Bracci-Laudiero et al., 1992).

NGF is mainly known as a neuronal survival factor, active on some peripheral fetal neurons and, in the CNS, on cholinergic neurons of basal forebrain (Varon et al., 1995). However, high affinity receptors for NGF were identified on pheripheral monocytes and/or T lymphocytes (Ehrhard et al., 1993), while the factor may be produced not only by astrocytes, but also by activated brain microglial cells (Mallat et al., 1989). Therefore, the involvement of NGF in the neuroimmune interactions which take place during an inflammatory reaction in the CNS appears probable, but remains elusive. In this general context, we have investigated whether LPS can induce NGF synthesis in newborn rat astrocytes, and analyzed the signalling pathway activated by LPS in these cells. Previous studies have shown that protein tyrosine-kinase activation and nuclear transcription factor NF-κB transduce LPS responses in monocytes or astrocytoma cells (Geng et al., 1993; Carlson and Aschmies, 1995). LPS strongly activates $p42^{mapk}$ and Raf-1, two essential components of the mitogen-activated protein (MAP) kinase signal transduction pathway (Willis and Nisen, 1996). Agents that elevate intracellular cyclic AMP (cAMP) have been shown to inhibit the MAP kinase pathway (Cook and McCormick, 1993; Wu et al., 1993) and to decrease LPS-induced IL-1β mRNA accumulation in human astrocytes (Willis and Nisen, 1995).

Our results show that LPS is a powerful inducer of NGF synthesis in astrocytes. The compound acts through a signal transduction pathway independent of protein kinase C, antagonized by cAMP-elevating agents, and involving presumably MAP kinase activation.

MATERIALS AND METHODS

Primary Cultures of Astroglial Cells

Cortical astroglial cells were derived from 1- to 2-day-old rats and cultured as previously described (Laviada et al., 1995). Cells were seeded at a density of 3×10^4 cells/cm^2 on plastic plates previously coated with 5 μg/ml *dl*-polyornithine in water. Cells were cultured for 3 weeks in basal medium consisting of a mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 (1:1, v/v), with 0.66% glucose, 5 μg/ml streptomycin, 5 U/ml penicillin, and supplemented with 10% fetal calf serum (FCS). Microglia-oligodendroglia-free astroglial cultures were obtained after 2 hours of orbital shaking. The primary cultures consisted of 95% astrocytes as judged by immunocytochemical staining of glial fibrillary acidic protein. Time course, dose response and Northern blot experiments were carried out in 57-cm^2 plates. The rest of the experiments were done in 6-well dishes.

Three days before the experiment, at day 21, the serum-containing medium was removed and cells were transferred to a chemically defined medium consisting of serum-free basal medium supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite.

RNA Analysis and Enzyme-Linked Immunosorbent Assay (ELISA)

After the indicated times of treatment, RNA extraction was carried out according to the LiCl/urea method. Northern blot analysis was also performed by standard procedures (Jehan et al., 1995). Glyoxal-treated RNA was fractionated in agarose gels, transferred to Hybond-N membranes by capillary blotting, and hybridized serially with a ^{32}P-labelled probe of mouse NGF cDNA. The NGF probe was the 917-bp NGF DNA cloned by Scott et al. (1983). Standardization of RNA loading was routinely controlled by hybridization of the blots with amyloid precursor protein cDNA probe (Shivers et al., 1988). Densitometric analyses were performed with PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA).

For extracellular NGF determination, cells supernatants were collected 24 hours after treatment as described in the text, diluted in one volume of phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 0.5% gelatin, and conserved frozen until quantitation. NGF released by the cells was assayed in triplicate, by a double-site ELISA, using a monoclonal anti-NGF antibody, coupled or not to β-galactosidase (Boehringer Mannheim, Indianapolis, IN), according to an experimental protocol described before (Laviada et al., 1995).

Other Chemicals

Tissue culture plastic wares were purchased from Nunc (Roskilde, Denmark), culture media from BioWhittaker (Verviers, Belgium), and FCS from JRH Biosciences (Sussex, England). *E. coli* 0127:B8 LPS *W* was from DIFCO Laboratories (Detroit, MI). It was dissolved and sonicated in PBS prior to utilization. Hybond-N membranes were from Amersham (Little Chaltfont, England). Apigenin, forskolin and bisidolylmaleimide were from Calbiochem (La Jolla, CA). 3-Isobutyl-1-methylxanthine (IBMX) and other reagents were from Sigma (St. Louis, MO).

RESULTS

Induction of NGF Expression and Secretion by LPS

Highly enriched astroglial cultures were exposed to increasing concentrations of *E. coli* LPS during 24 hr, and secreted NGF was determined in the supernatant of cultured cells. As shown in Figure 1, LPS treatment increased the amount of extracellular NGF in a dose-dependent manner. Maximal stimulation (10-fold over control) was observed at 2 μg/ml. In addition, Northern blot analysis of total cellular RNA was used to study the effect of LPS on NGF mRNA levels. A similar concentration-dependent effect of LPS was observed on NGF mRNA accumulation (Fig. 2).

Primary cultures of astrocytes were treated with a maximal dose of LPS (2 μg/ml) and extracellular NGF and NGF mRNA accumulation were assessed after various time periods. Figure 3 shows that there was no increase in extracellular NGF concentration after 8 hr of treatment. In contrast, a striking enhancement was noticed after 24 hr. Maximal NGF secretion occurred in cells treated for 72 hr with LPS (17-fold increase over basal value). Densitometric analysis of Northern blots presented in Figure 4 indicated that a 4-fold increase in NGF transcripts took place after 8 hr of treatment, whereas maximal levels of NGF mRNA were reached after 24 hr of treatment (8-fold increase).

To assess whether LPS induction was dependent on DNA transcription and RNA translation, we employed an inhibitor of DNA transcription, actinomycin D (Act D), and an inhibitor of protein synthesis, cycloheximide (CHX). Results summarized in Table I show that treatment with either 2.5 μM Act D or 5 μM CHX completely blocked the augmentation of NGF secretion induced by LPS.

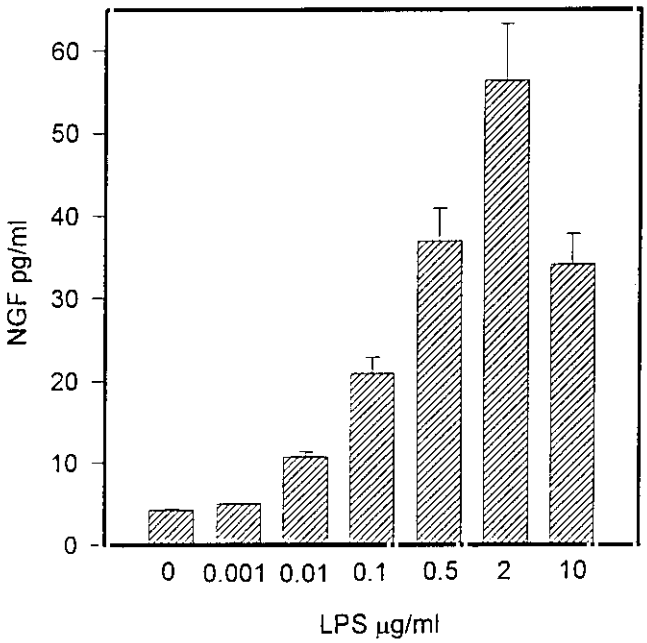

Fig. 1. Nerve growth factor (NGF) secretion by astroglial cells treated with increasing concentrations of lipopolysaccharide (LPS). Primary cultures of astrocytes, 3 weeks old, were incubated with increasing concentration of LPS during 24 hr. At this moment, NGF released in the supernatants was assayed with a double-site ELISA. Data are means ± S.E.M. of four independent experiments. Each determination was in triplicate. Maximal NGF secretion was obtained with 2 μg/ml LPS.

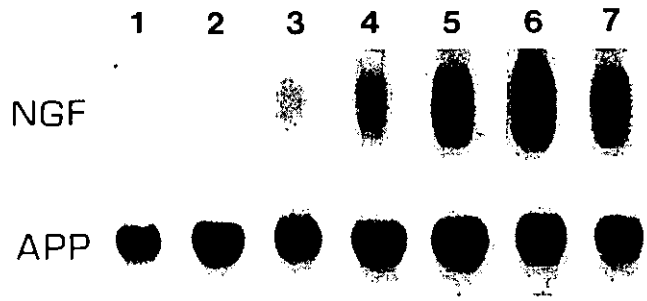

Fig. 2. Northern blot analysis of NGF mRNA accumulation induced by increasing concentration of LPS. Cells were treated with increasing concentrations of LPS. **Lane 1,** untreated cells; **lane 2,** 0.001 μg/ml; **lane 3,** 0.01 μg/ml; **lane 4,** 0.1 μg/ml; **lane 5,** 0.5 μg/ml; **lane 6,** 2 μg/ml; **lane 7,** 10 μg/ml. After 24 hr incubation, total RNA was isolated, electrophoresed, blotted and serially hybridized with a radiolabelled NGF and APP cDNA probes. Essentially identical results were obtained in three independent experiments.

PKC and Tyrosine Kinase Inhibition Do Not Affect the Induction of NGF Secretion by LPS

In an attempt to study the pathways involved in the control of NGF synthesis by LPS in astrocytes, cells were treated with bisidolylmaleimide (BIM), a highly specific inhibitor of PKC, prior to the incubation with LPS. Classical PKCs are strongly activated by phorbol esters as phorbol 12-myristate 13-acetate (PMA), which is a potent inducer of NGF gene in astrocytes (Neveu et al., 1992). We have previously described that treatment with the PKC inhibitor BIM abolished PMA-induced NGF (Laviada et al., 1995). Treatment of astrocytes with BIM did not affect LPS-induced NGF secretion (Table I), indicating that the signal transduction pathway triggered by LPS was PKC-independent.

Tyrosine kinase inhibitors have been shown to block some of the LPS effects. To study whether activation of tyrosine kinases was involved in NGF induction by LPS in astroglial cells, we employed the inhibitors tyrphostin AG126 and genistein. As shown in Table I, neither tyrphostin AG126 nor genistein affected LPS-stimulated secretion of NGF.

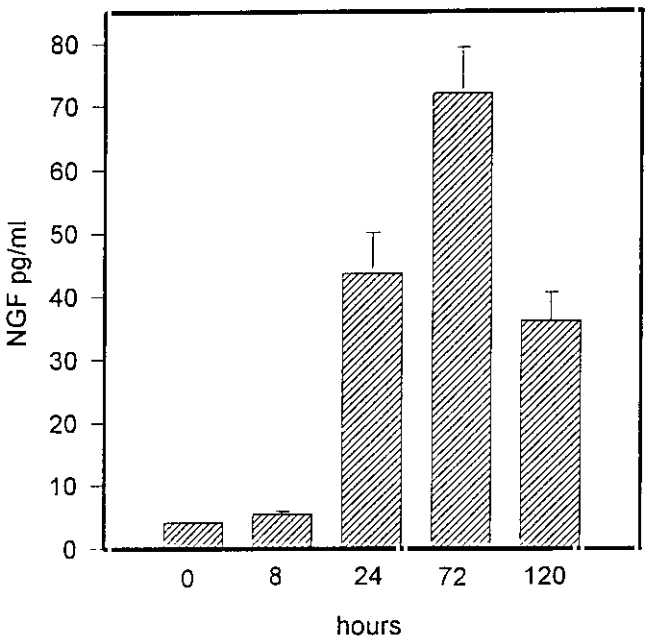

Fig. 3. Time course of NGF secretion after treatment with LPS. Primary astrocyte cultures were incubated in the presence of 2 μg/ml LPS. At the indicated times, cell-secreted NGF was assayed with a double-site ELISA assay. Data are mean ± S.E.M. of two independent experiments with determinations in triplicate. Maximal induction occurred at 72 hours of treatment.

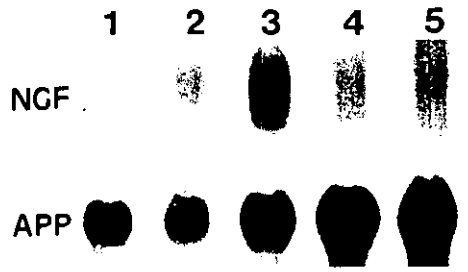

Fig. 4. Time course of NGF mRNA accumulation in astroglial cells treated with LPS. Cells were treated at the indicated times with 2 μg/ml LPS and NGF mRNA accumulation assessed by Northern blot. **Lane 1,** 0 hours; **lane 2,** 8 hours; **lane 3,** 24 hours; **lane 4,** 72 hours; **lane 5,** 120 hours. Essentially identical results were obtained in two independent experiments.

TABLE I. Effect of Treatment With Different Agents on LPS-Induced NGF Secretion by Astroglial Cells*

Addition	NGF pg/ml
Control	16.2 ± 3.0
LPS 2 μg/ml	109.3 ± 6.6
LPS 2 μg/ml + Act D 2.5 μM	9.9 ± 2.5
LPS 2 μg/ml + CHX 5 μM	12.0 ± 1.3
LPS 2 μg/ml + BIM 2 μM	104.5 ± 9.4
LPS 2 μg/ml + Tyr AG126 100 μM	105.0 ± 26
LPS 2 μg/ml + genistein 100 μM	103.6 ± 15
LPS 2 μg/ml + FK 10 μM	25.6 ± 10.7
LPS 2 μg/ml + IBMX 100 μM	21.7 ± 7.5
LPS 2 μg/ml + API 25 μM	22.9 ± 2.2

*Primary cultures of astrocytes were incubated in the presence of the indicated agents 1 hour prior to LPS treatment for 24 hours. NGF in the supernatants was assayed with a double-site ELISA. Data are means ± S.E.M. of three independent experiments with determinations in triplicate. Agents alone, which had no effect on NGF secretion, have been omitted to clarify the table.

Abbreviations: Act D, actinomycin D; CHX, cycloheximide; BIM, bisindolylmaleimide; Tyr AG126, tyrphostin AG126; FK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; API, apigenin.

Agents Elevating cAMP Levels Inhibit LPS-Induced Production of NGF

The effect of raising intracellular cAMP levels on LPS-induced NGF release by astroglial cells was determined by treating cells with either forskolin (FK) or IBMX. FK or IBMX alone had no effect on NGF secretion. However, a 1-hr preincubation with these agents led to a complete inhibition of LPS-stimulated secretion of NGF. Cell treatment with the cAMP analogue dibutyryl-cAMP exerted the same inhibitory effect (data not shown). To confirm the antagonistic action of cAMP on the induction of the NGF gene by LPS, Northern blot analyses were carried out under the same treatment conditions. Figure 5 shows that both FK and IBMX do completely block the accumulation of NGF transcripts which occurs in response to a LPS treatment.

Stimulation of NGF Production by LPS Is Inhibited by Apigenin

To further examine the pathway by which LPS induces NGF synthesis and secretion by astroglial cells, the plant flavonoid apigenin (API) was used. Cells were preincubated with apigenin for 1 hr, and then treated with LPS. Under such conditions, LPS failed to induce any augmentation of the amount of cell secreted NGF (Table I). The compound also blocked the NGF mRNA accumulation in the cells (Fig. 6). The dose-response curve shows that the IC_{50} value for apigenin inhibition was approximately 10 μM (Fig. 7).

DISCUSSION

The present study shows that LPS is a potent activator of NGF secretion in primary cultures of cortical astrocytes. LPS induced NGF synthesis and secretion by astroglial cells in a concentration-dependent manner. A dose of 10 ng/ml was already effective but the strongest response was obtained with a dose of 2 μg/ml LPS. At this concentration, LPS induced an accumulation of NGF mRNA and NGF proteins which reached their maximal levels after 24 hr and 72 hr of treatment, respectively. The protein secreted in the extracellular medium was in-

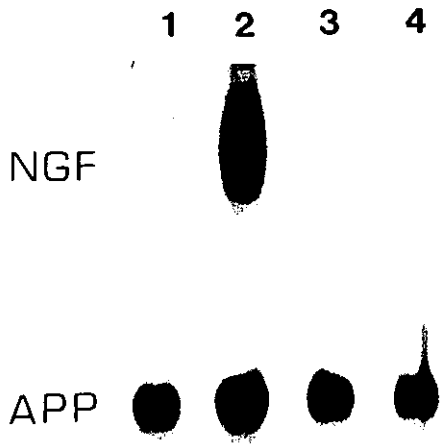

Fig. 5. cAMP-elevating agents counteract NGF mRNA accumulation induced by LPS. Astrocyte cultures were treated with no additions (**lane 1**) or with 2 μg/ml LPS alone (**lane 2**) or in the presence of either forskolin (FK) 10 μM (**lane 3**) or 3-isobutyl-1-methylxanthine (IBMX) 100 μM (**lane 4**). FK and IBMX were added 1 hour prior to LPS treatment. Essentially identical results were obtained in two independent experiments.

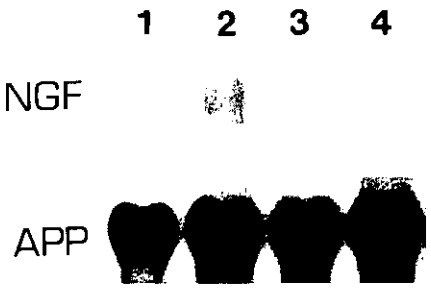

Fig. 6. Apigenin inhibits NGF mRNA accumulation in astroglial cells induced by LPS. Primary cultures were treated with no additions (**lane 1**) or with 2 μg/ml LPS (**lane 2**), 25 μM apigenin (**lane 3**) or 2 μg/ml LPS plus 25 μg/ml apigenin (**lane 4**). The latter was added 1 hour prior to LPS treatment. Essentially identical results were obtained in two independent experiments.

creased by a factor of 17-fold, which is considerable. The effect of LPS required both protein synthesis and DNA transcription, as it was abrogated by inhibition of either biosynthetic steps, using CHX and Act D.

LPS induction of NGF by astrocytes may be the result of a complex process comprising the various effects of LPS on those cells, as well as on other cell types that might be present in the primary culture. However, the experimental procedure used in the present work, which provides highly enriched astrocytic cultures, points to a direct effect of LPS on NGF synthesis and secretion by astrocytes. Nevertheless, stimulation of NGF synthesis may not result from a direct action of LPS on astrocytes, but from autocrine loops. Other products released by

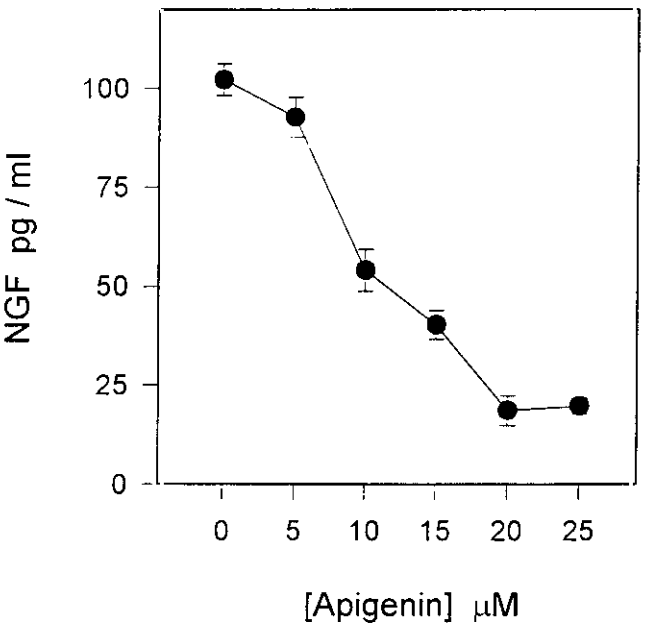

Fig. 7. Dose response inhibition of LPS-inducted NGF secretion by apigenin. Astrocyte cultures were incubated with 2 μg/ml LPS and the indicated doses of apigenin for 24 hours and NGF secreted to the supernatants was assayed with a double-site ELISA assay. Data are means ± S.E.M. of two independent experiments with determinations in triplicate.

astrocyte in response to LPS could enhance the expression of the NGF gene. This is the case of inflammatory cytokines TNFα, TGFβ and IL-1β, which have been described as inducers of NGF synthesis (Spranger et al., 1990; Pshenichkin et al., 1994; Plüss et al., 1995; Hattori et al., 1996). However, induction of cytokines by LPS has been shown to occur through protein tyrosine kinase activation (Geng et al., 1993). Tyrphostin AG126 has been shown to prevent LPS toxicity in vivo and blocks LPS-stimulated TNFα and nitric oxide (NO) production by macrophages (Novogrodsky et al., 1994; Vanichkin et al., 1996). Genistein has also been implicated in the inhibition of the synthesis of IL-6 stimulated by IL-1β (Carlson and Aschmies, 1995). Our results did not evidence any effect of the tyrosine kinase inhibitors tyrphostin AG126 and genistein on the LPS-induced NGF production. Therefore, our data suggest that the regulation of the expression of the NGF gene elicited by LPS is not mediated by TNFα, IL-1, IL-6 or NO production.

To further study the mechanism triggered by LPS and leading to a stimulation of NGF production by astrocytes, we examined whether agents able to increase intracellular cAMP can inhibit the effect of LPS. The role of cAMP and protein kinase A in NGF regulation by astroglial cells is still not clearly defined. Depending on the cell culture conditions, the growth state of cells and the experimental model, different results have been

obtained. While under certain conditions, forskolin, catecholamines or cAMP analogues stimulated NGF synthesis, the compounds were found by others to be ineffective or able to counteract the promotory action of several inducers of NGF synthesis (Hahn et al., 1994; Jehan et al., 1995). Our results fit with this scheme, since they show that FK and IBMX block the stimulatory effect of LPS, both at the level of NGF mRNA accumulation and cell-secreted protein. FK and IBMX have been involved in the blockade of the MAP kinase pathway in epidermal growth factor (EGF)-stimulated cells through a cAMP-dependent increase in Raf-1 phosphorylation, which prevents Ras-dependent activation of Raf-1 (Cook and McCormick, 1993; Wu et al., 1993). Our data indicate that apigenin, a proposed inhibitor of the MAP kinase pathway, also inhibited LPS-induced NGF synthesis. Apigenin has been demonstrated to reverse the v-H-ras-transformed phenotype of NIH 3T3 cells through the inhibition of the MAP kinase-associated signal transduction pathway (Kuo and Yang, 1995). Our result suggests that LPS triggers in astroglial cells a MAP kinase-dependent mechanism which promotes the activation of the NGF gene. It is noteworthy that activation of the MAP kinase cascade by LPS, and its inhibition by agents that elevate cell levels of cAMP, was recently reported in a human astrocytic cell line, U-373 MG (Willis and Nisen, 1995).

Two general mechanisms appear to promote the expression of the NGF gene in astrocytes. The first one operates through the activation of PKC, and is triggered by serum and diacylglycerol (D'Mello and Heinrich, 1990; Neveu et al., 1992). The other one acts through the sphingomyelin pathway, and is thought to be switched on by vitamin D_3 (Neveu et al., 1994). We have recently described that the breakdown of phosphatidylcholine by phospholipase C may activate both mechanisms (Laviada et al., 1995). Results shown in the present report indicate that the regulation of NGF production by LPS in astrocytes seems to be independent of PKC activation, and therefore, LPS could control NGF synthesis by the second mechanism. Bacterial LPS has been shown to trigger similar cellular responses to TNFα and IL-1β, which are known to initiate a signalling pathway involving sphingomyelin hydrolisis (Kolesnick and Golde, 1994; Hannun et al., 1996) and Raf-1 activation (Belka et al., 1995). Recent studies suggest that LPS is recognized by the same intracellular molecules that bind the second messenger ceramide (Wright and Kolesnick, 1995). Furthermore, LPS can stimulate a ceramide-activated protein kinase in HL-60 cells (Joseph et al., 1994) which appears capable of catalyzing the phosphorylation of Raf (Yao et al., 1995). This suggests that the MAP kinase cascade is involved in the sphingomyelin pathway. Therefore, our results indicate that LPS stimulates NGF synthesis in astrocytes by a mechanism that seems to be closely related to the pathway activated by sphingomyelinase and which is independent of PKC. The data raise the possibility that stimulation of the synthesis of NGF is a direct consequence of LPS action on astrocytes, rather than an indirect response elicited by LPS-induced cytokines such as IL-1β, TNFα or TGFβ.

NOTE ADDED IN PROOF

We have recently observed that LPS induces a 3-fold stimulation of p42/p44 MAPK in primary cultures of astrocytes.

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RESULTADOS Y DISCUSIÓN

Regulación de la producción de NGF por el TNFα. Papel del NF-κB

Los resultados expuestos ponen de manifiesto el efecto inductor del TNFα en la síntesis de NGF. Del estudio de la regulación del NGF por el TNFα se desprende que este ejerce su efecto por un mecanismo diferente, y probablemente independiente, de la generación de ceramidas por acción de la SMasa. La síntesis de NGF inducida por TNFα y LPS es independiente de cPKC, de forma similar al efecto promovido por ceramidas. Sin embargo, la inducción de NGF por ceramidas parece estar mediada por la activación de la cascada de MAPK, mientras que el efecto del TNFα es independiente de este sistema de fosforilación. Esta constituye la primera observación de que ambos mediadores, TNFα y ceramidas, ejercen su efecto de manera independiente. La ausencia de hidrólisis de SM y de generación de ceramidas por TNFα en astrocitos, indica que esta citoquina no activa la síntesis de NGF a través de la SMasa. Los resultados descritos indican la existencia de otros mecanismos endógenos distintos de la acción de la SMasa, como puede ser la estimulación de la síntesis *de novo* o la inhibición de su degradación para incrementar los niveles intracelulares de ceramidas (Bose et al., 1995; Biewlaska et al., 1996; Paumen et al., 1997). Es importante destacar que una proteína viral de HIV-1 favorece la activación de la SMasa por el TNFα, lo que revela que este sistema de transducción de señales es funcional en células gliales en una situación de infección vírica (Richard et al., 1996). Experimentos realizados *in vivo* muestran como la inyección de otra proteína viral de HIV-1 aumenta la expresión de NGF *in vivo* (Bagetta et al., 1996). El conjunto de estas observaciones sugiere que el aumento de los niveles intracelulares de ceramida puede ser importante en los astrocitos reactivos, induciendo entre otras respuestas un aumento en la síntesis y secreción de NGF.

Por otra parte se observa que la inducción del NGF se inhibe por el óxido de fenilarsina, inhibidor de tirosina fosfatasas. Este modulador bloquea la activación del factor de transcripción nuclear NF-κB, así como la degradación de su subunidad citosólica inhibidora IkBα. Parece por tanto que NF-κB, está implicado en la regulación de la expresión de NGF por TNFα. El estudio del promotor del gen de NGF indica la existencia de una posible secuencia reguladora que podría unir este factor de transcripción (Jehan et al.1993). Sin embargo, los experimentos realizados demuestran que, al menos *in vitro,* el oligonucleótido correspondiente a esta secuencia no es capaz de unir el factor de transcripción. Por tanto la expresión del gen

de NGF inducida por TNFα depende de NF-κB pero de forma indirecta y no por la unión directa al promotor del gen de NGF. La inhibición parcial que ejerce el cAMP sobre la secreción de NGF inducida por TNFα no puede ser atribuida en este caso a una inhibición de la cascada de MAPK, ya que este sistema de fosforilación aparentemente no esta implicado (Kurino et al., 1996, Willis y Nisen, 1996). Sin embargo, la inhibición parcial de la activación de NF-κB por el aumento en los niveles de cAMP intracelular (Pahan et al., 1997), constituye una alternativa para explicar el efecto inhibidor del cAMP sobre la síntesis de NGF.

Regulación de la secreción de NGF por LPS

En lo que se refiere al LPS, los resultados obtenidos ponen de manifiesto el potente efecto inductor del LPS (*E.coli*) en la síntesis y secreción del NGF por astrocitos en cultivo primario. Los inhibidores de actividad tirosina quinasa empleados no bloquean la secreción de NGF inducida por LPS, lo que sugiere una activación directa del LPS y no un efecto indirecto mediado por la secreción de citoquinas pro-inflamatorias, inductores de la secreción de NGF (Carman-Krzan et al., 1991; Hattori et al., 1993; Pshenichkin et al., 1994). Para profundizar en este aspecto, se ha estudiado también el posible papel del óxido nítrico (NO) en la inducción de NGF por LPS. El NO es un importante mensajero paracrino regulador de diferentes situaciones patofisiológicas en el SNC (Murphy et al., 1993; Schmidlin y Wiesinger, 1995). La exposición de astrocitos a LPS o a citoquinas pro-inflamatorias activa la generación de NO a través de la NO sintasa inducible, por ello resulta factible que la generación de NO constituya un mecanismo mediador de la regulación de la síntesis de NGF. Los resultados obtenidos (no incluidos) con aminoguanidina (inhibidor de la NO sintasa constitutiva e inducible), y L-NAME (inhibidor competitivo de la NO sintasa constitutiva) ponen de manifiesto que este sistema de transducción de señales carece de efecto en la regulación por LPS de los niveles de NGF sintetizado. Por tanto, la secreción de NGF inducida por LPS es independiente de la producción de NO y posiblemente también de citoquinas pro-inflamatorias. En este contexto, es importante destacar que la estimulación de la vía de las ceramidas puede simular algunas de las respuestas celulares inducidas por el LPS (Barber et al., 1996).

La inhibición a través de la vía del cAMP de la secreción de NGF inducida por LPS pone de manifiesto la existencia de mecanismos de regulación que permiten un control preciso de la síntesis de este factor neurotrófico. Los datos obtenidos con

apigenina, inhibidor de la cascada de MAPK, fueron confirmados posteriormente con el uso de un inhibidor sintético altamente específico de MEK, denominado PD 098059 (Alessi et al., 1995). El tratamiento simultáneo de los astrocitos en cultivo primario con PD 098059 y la dosis máxima de LPS disminuye en un 50% la estimulación máxima producida por LPS en ausencia del inhibidor. Puesto que el LPS es un importante activador de NF-κB, el efecto inhibidor del cAMP en la síntesis de NGF inducida por LPS puede, por tanto, ser explicado por una inhibición de la cascada de MAPK, como por la inhibición de la activación de NF-κB (Pahan et al., 1997).

III- DISCUSIÓN GENERAL Y CONCLUSIONES

III- DISCUSIÓN GENERAL Y CONCLUSIONES

El funcionamiento del SNC depende del equilibrio funcional entre sus distintos componentes celulares y de una adecuada integración y adaptación del sistema a unas condiciones externas variables. Los astrocitos constituyen un importante centro de coordinación funcional en el SNC, a través de la expresión de neurotrofinas, proteínas de adhesión, citoquinas y factores de crecimiento que regulan el funcionamiento neuronal, su desarrollo y la plasticidad neuronal. Además, los astrocitos son activadores de respuestas de emergencia ante procesos de lesión o infección mediante su contribución al proceso de gliosis. En este contexto, los niveles locales de neurotrofinas, como el NGF, favorecen el crecimiento de determinadas poblaciones neuronales en momentos específicos del desarrollo. Un descenso "programado" en los niveles de NGF puede ser importante para restringir, eliminar o modificar poblaciones neuronales locales. El propio NGF puede desencadenar procesos de apoptosis, necesarios durante el desarrollo del sistema nervioso. La síntesis neuronal de NGF en el SNC puede ser sustituida por la síntesis glial, siendo los astrocitos las células no neuronales más abundantes del SNC. La inducción de la síntesis de NGF por astrocitos activados puede proporcionar el suficiente suministro de NGF en el SNC en determinadas situaciones.

La síntesis de NGF está muy regulada y controlada por las condiciones extracelulares. In vivo, en una situación fisiológica normal y en el SNC adulto, la expresión del NGF por astrocitos esta reprimida pero la activación astrocitaria induce su expresión. Los resultados mostrados profundizan en el conocimiento de los sistemas de transducción de señales que activan la síntesis y secreción de NGF por astrocitos en cultivo primario. La ruta de la SMasa y la consiguiente generación de ceramidas presenta un alto grado de similitud con vías de transducción de señales clásicas y mejor conocidas como la generación de DAG por activación de diferentes fosfolipasas. El DAG y la ceramida son estructuralmente semejantes y una vez generados por la fosfolipasa correspondiente permanecen embebidos en la membrana plasmática. De manera análoga al DAG, que actúa dirigiendo a algunos de los tipos de PKC a la membrana donde se activan, la ceramida podría actuar como punto de unión dirigiendo su(s) proteína(s) efectora(s) hacia la membrana, lo que implicaría su activación. Las ceramidas pueden por tanto modular la actividad de sus efectores induciendo cambios conformacionales de la proteína, modificando su localización subcelular por translocación y favoreciendo el acceso a sus substratos. La existencia de una modulación diferencial de PKC ζ y Raf-1 por los análogos de ceramida de

diferente longitud del grupo acilo, puede explicarse desde el modelo del grupo alquilo protuberante (Krönke, 1997). Según este modelo, el grupo alquilo de la SF sería reconocido por las proteínas efectoras de los esfingolípidos, mientras el resto acilo sería el responsable de la inserción en la membrana plasmática. Si esto fuera así, la C2-ceramida podría ser reconocida por las mismas proteínas efectoras que ceramidas de mayor longitud, pero carecería de la capacidad de dirigirlas a la membrana plasmática.

La inducción observada de NGF por acción de la PC-PLC, puede reflejar el efecto regulador sobre la síntesis de NGF de los sistemas de control de proliferación celular, dado que la hidrólisis de PC constituye uno de sus mecanismos de regulación. El efecto promovido por la PC-PLC confirma, además, la importancia de la generación de mensajeros lipídicos en la regulación de la funcionalidad astrocitaria. La inducción de la síntesis de NGF por la acción de la PC-PLC posee características comunes con el efecto observado por la acción de la SMasa exógena y las ceramidas permeables, lo que hace pensar que ambos efectos están relacionados de forma sucesiva y coordinada. El trabajo realizado en la presente tesis doctoral constituye un ejemplo de la importancia de los mensajeros lipídicos en la regulación de la funcionalidad celular. Glicerolípidos y esfingolípidos son importantes reguladores de sistemas de fosforilación de proteínas y la regulación cruzada que ejercen entre sí aumenta la complejidad de los mecanismos de control posibles.

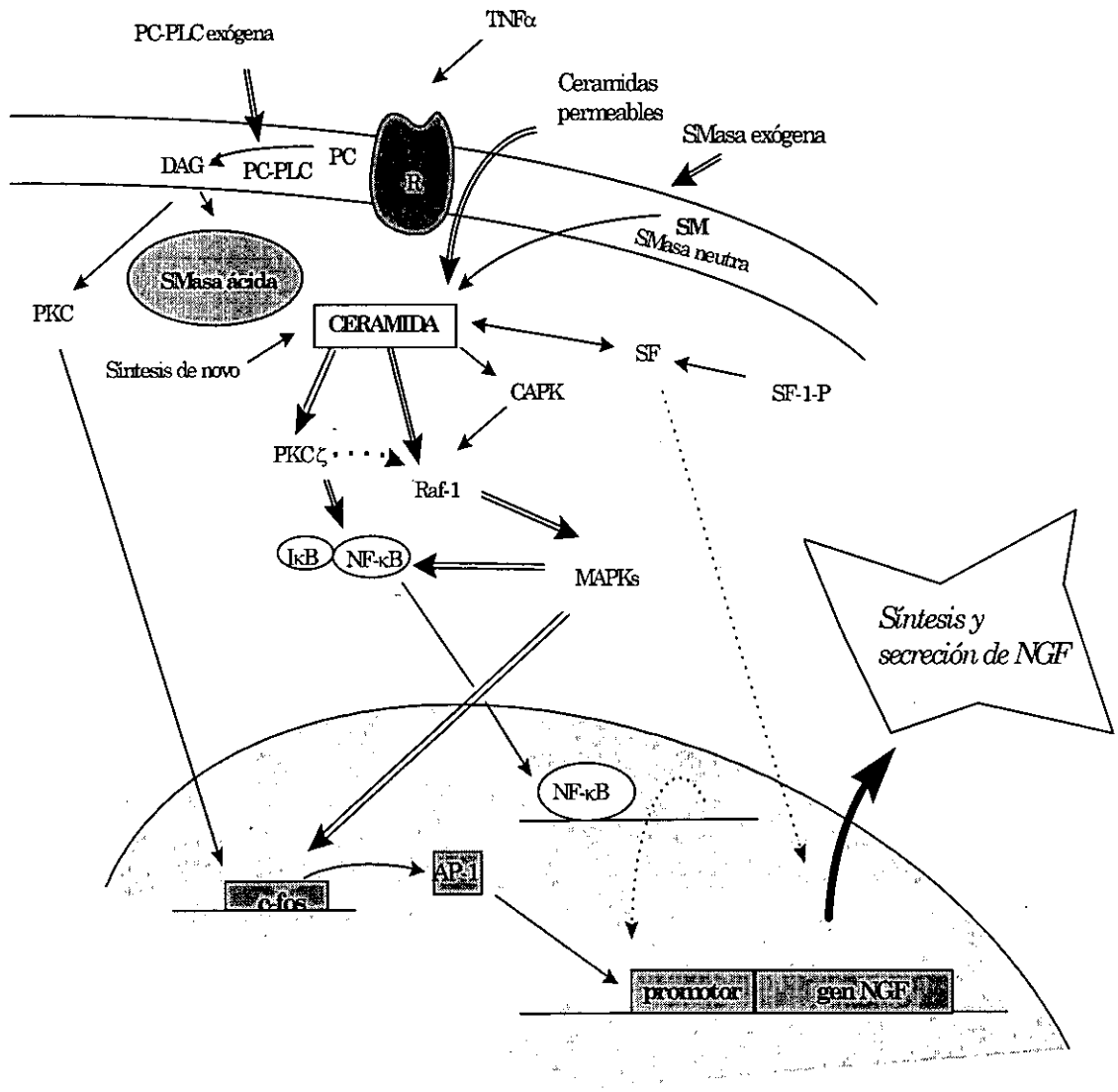
El LPS, uno de los principales activadores de la respuesta inmune e inflamatoria ante una situación de infección bacteriana, induce un drástico aumento en los niveles de NGF, sintetizado por astrocitos en cultivo primario. El efecto regulador del LPS puede tener diferentes lecturas pero todas ellas relacionadas con los resultados anteriormente descritos. La vía de las ceramidas puede ser activada por la exposición celular a endotoxinas (Hannun, 1996), y se ha propuesto que el LPS puede ejercer alguno de sus efectos por analogía estructural con las ceramidas activando efectores similares a los activados por el aumento en los niveles intracelulares de ceramidas como la CAPK (Wright y Kolesnick, 1995). Las características comunes de la estimulación de la síntesis de NGF por el LPS y ceramidas sugieren que ambos efectos podrían estar relacionados con la inducción de NGF durante una situación inflamatoria o de lesión.

El TNFα, otro activador característico pro-inflamatorio de la vía de la SMasa-ceramidas (Hannun, 1996), promueve sin embargo un incremento en la síntesis de NGF con algunas diferencias con la inducción observada por el aumento de los niveles

intracelulares de ceramida, lo que sugiere que ejerce su acción de manera independiente de la hidrólisis de SM. En efecto, el TNFα no induce un aumento en los niveles intracelulares de ceramida a través de la hidrólisis de SM. La inducción de NGF por un aumento en los niveles de ceramida se puede explicar por la activación de la ruta de la SMasa por otros activadores de este sistema de señalización celular, o por un aumento en los niveles intracelulares debido a la regulación de su síntesis o degradación.

Respecto a los sistemas de fosforilación implicados, los resultados expuestos constituyen la primera referencia, de la que tenemos constancia, que asigna un papel a la cascada de las MAP quinasas en la regulación de la expresión del NGF. La importancia de este sistema de fosforilación en la regulación de la síntesis de NGF se ve confirmada al observarse que distintos activadores de la síntesis de NGF dependen en mayor o menor medida de la actividad MAPK. La proteína quinasa PKC ζ es una de las dianas de la acción de la PC-PLC y las ceramidas en los astrocitos, modificando su localización subcelular y su grado de fosforilación. La localización perinuclear de PKC ζ inducida por la activación de los astrocitos puede estar relacionada con efectos de regulación de la expresión génica, como ocurre también en otros tipos celulares gliales y neuronales (Carlson y Hart, 1996; Wooten et al., 1997).

La activación de PKC ζ por ceramidas puede ocurrir por interacción directa, además las ceramidas son capaces de activar Raf-1, lo que se relaciona con el efecto activador de PKC ζ sobre MEK y MAPK. Recientemente se ha descrito que PKC ζ es capaz de activar e interaccionar de forma directa con Raf-1, lo que explicaría un mecanismo de activación de MAPK independiente de Ras desencadenado por la activación de la PC-PLC (Van Dijk et al., 1997a). Estas observaciones junto con los resultados mostrados permiten proponer un modelo en el que en los cultivos primarios de astrocitos puede existir una relación entre la activación de la PC-PLC y la generación de ceramidas por activación de la SMasa. Las ceramidas generadas son capaces de incrementar la actividad MAPK, pudiendo atribuirse esta activación a su efecto regulador sobre PKC ζ y Raf-1. Este sistema de transducción de señales podría constituir un importante sistema de regulación de la síntesis de NGF en situaciones de activación astrocitaria como la que ocurre durante el proceso de gliosis.



Esquema de los principales aspectos estudiados en la presente memoria de tesis doctoral

IV- BIBLIOGRAFÍA

V- BIBLIOGRAFÍA

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