A Ras fehérjék szerepe a sejten belüli jelátvitelben

Ph.D. tézisek

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BEVEZETÉS ÉS CÉLKITÜZÉSEK:

1.Bevezetés:

A Ras fehérjék a G proteinekhez hasonló funkciót látnak el a sejtben, vagyis a sejtmembrán receptorokat a sejten belüli másodlagos hírvivô rendszerekhez kapcsolják. A Ras proto-onkogének a plazmamembrán belsô felszínén találhatók, specifikusan és nagy affinitással kötnek GDP-t (inaktív forma) és GTP-t (aktív forma), valamint GTP bontó aktivitással is rendelkeznek. Ezen kétféle biokémiai folyamat fiziológiás szabályozása két különbözô protein mûködése által valósul meg. Egyikük a GTP-áz aktiváló protein (GAP), másikuk a GDP-GTP cserét elôsegítô fehérje (GRF=guanine-nucleotide releasing factor).

A proto-onkogén forma pontmutációk révén aktív onkogénné alakulhat. Az aktiváló mutációk vagy a GTP-áz aktivitást csökkentik, vagy a GDP-GTP cserét fokozzák, vagy mindkettôre hatással vannak. Ezek következménye az aktív Ras-GTP komplex felszaporodása és szabályozatlan, konstitutív Ras aktivitás létrejötte.

Ha a GTP-áz aktivitás nô és/vagy a GDP-GTP csere csökken, a Ras elveszti biológiai aktivitását. Erre példa a Ha-Ras Asn-17, amelyet G.M. Cooper laboratóriumában sikerült elôállítani 1988-ban. Ez a mutáns Ras a 17-es helyen valin helyett aszparagint tartalmaz (Ha-Ras Asn-17), ezáltal a GTP iránti affinitása kb. 30-adára csökken, miközben a GDP-t továbbra is változatlan affinitással köti. A mutáns Ras a kolóniaképzôdés mérése során gátolta az NIH 3T3 fibroblasztok szaporodását, azaz nemcsak elvesztette aktivitását, hanem domináns gátló molekulaként mûködött a biológiai rendszerekben.

Ezt felismerve Cooper és munkatársai NIH 3T3 egér fibroblasztokat és PC12 patkány feokromocitóma sejteket stabilan transzfektáltak Ha-ras Asn-17-tel. A domináns gátló mutáns Ras expressziója az NIH 3T3 sejtvonalakban gátolta a szaporodást és tranziens transzfekció esetén a korai válasz gének indukcióját is.

PC12 sejtekben a sejtszaporodás változatlan ütemû maradt, de az egyébként differenciációt okozó NGF (idegsejt növekedési faktor) hatástalannak mutatkozott. A domináns negatív rassal transzfektált PC12 sejtek két csoportját lehetett elkülöníteni a mutáns Ras expressziójának mértéke szerint. Alacsony expresszió mellett gátolt volt az NGF által okozott

differenciáció, holott a korai válasz gének indukciója normális maradt. A magas expressziójú sejtekben a korai válasz gének NGF-indukciója is erôteljesen csökkent, a Ras mûködés fokozottabb gátlása miatt..

A fentebb leírt domináns negatív rassal transzfektált NIH 3T3 és PC12 sejtvonalakat használtam az alábbi célkitûzések megvalósításához.

2. Célkitûzések:

Kísérleteink során a Ha-ras Asn-17-tel stabilan transzfektált NIH 3T3 és PC12 sejtek segítségével vizsgáltuk a Ras protein sejtosztódásban és differenciációban betöltött szerepét..

Az **NIH 3T3 sejtek** vizsgálatakor a PC-PLC (foszfatidilkolin specifikus foszfolipáz C) enzimet tanulmányoztuk behatóan. Ennek oka az a korábbi megfigyelés volt, hogy bakteriális PC-PLC enzimpreparátum jelenléte a szövettenyészeti táptalajban fokozza a DNS szintézist, továbbá a Ras-transzformált fibroblasztokban intenzívebb a foszfatidilkolin hidrolízise. Ezek a vizsgálatok felvetették annak a lehetôségét, hogy a Ras és a PC-PLC kapcsolatba kerülnek egymással a jelátviteli folyamatban. Kísérleteink során az alábbi kérdésekre kerestük a választ:

1. Hogyan viszonyul egymáshoz a Ras protein és a PC-PLC enzim a jelátvitelben?

Munkánk kezdetén ismert volt, hogy mindkettô mitogén hatású, de kapcsolatukra vonatkozóan direkt bizonyíték nem állt még rendelkezésre az irodalomban.

2. Hogyan viszonyul a PC-PLC enzim a többi Ras-függô jelátviteli molekulához?

Ez a kérdés azt követôen került vizsgálataink középpontjába, hogy sikerült bizonyítani azt, hogy a PC-PLC egyes emlôs sejtekben a Ras effektora . A válaszadást megkönnyítette az a megfigyelésünk, hogy a PC-PLC génjével végzett kotranszfekciós kísérletekben a PC-PLC áthidalta a domináns gátló Ras hatását, de a domináns gátló Rafét nem. Igy kérdésfelvetésünk az alábbira módosult:

Hogyan viszonyul a PC-PLC enzim a Raf kinázhoz a jelátvitelben?

A **PC12 sejteken** végzett vizsgálataink azon a felismerésen alapultak, hogy a rendelkezésre álló Ha-ras Asn-17-tel transzfektált sejtek viselkedése a mutáns Ras expressziójának mértékétôl függ. Ez két, egymástól eltérô Ras-függô út lehetôségét vetette fel, amelyek közül mindkettô gátolt a magas expressziójú, de csak az egyik az alacsony expressziójú sejtekben. Ezért elsôként a legismertebb jelátviteli mechanizmusok (cAMP, Ca^{++}, protein kináz C) viselkedését tanulmányoztuk a Ras mûködésben gátolt transzfektánsokban. Ez vezetett annak a leírásához, hogy az NGF-dbcAMP ill. az NGF-ionomicin kezelési kombinációk szinergizmusban vannak egymással és áthidalják a mutáns Ras gátló hatását. Ennek a jelenségnek a segítségével NGF által indukált, de a Rastól független út vagy utak jelenlétét mutattuk ki, amelyek a szinergizmusban az "NGF részt" képviselik.

Ezen Ras-független, de NGF által indukált út, valamint a korábban említett Ras-függô utak vizsgálata az alábbi kérdéscsoportokra tagolható:

1. Hogyan mûködnek az alacsony expressziójú PC12 transzfektánsokban a már ismert Ras-függô utak, úgymint a Raf kinázok, a MAP kinázok, az eIF-4E (eukarióta iniciációs faktor 4E) és a PC-PLC (foszfatidilkolin specifikus foszfolipáz C)?

2. Hogyan függnek a Ras mûködés gátlásától az olyan NGF-fel indukálható utak, amelyeknek Rashoz való viszonya nem ismert? Ezek a PI-3-kináz (foszfatidilinozitol-3-kináz), a Na^{+} csatorna fehérjék és a PI-PLC (foszfatidilinozitol specifikus foszfolipáz C) gamma voltak.

3. Hogyan mûködik a normál ill. a transzfektáns PC12 sejtekben néhány olyan szignál, amely a kísérletek végzésének idôpontjában akkori ismereteink szerint Rastól független volt vagy nem volt pontosan definiálva? Ezek közül a cAMP, a Ca ion és a protein kináz C (PKC) Rashoz és egyéb jelátviteli utakhoz fûzôdô kapcsolatával foglalkoztunk.

EREDMÉNYEK ÉS MEGBESZÉLÉS:

1. A foszfatidilkolin specifikus foszfolipáz C (PC-PLC) aktiválása Ras-függô folyamat az NIH 3T3 sejtekben:

Korábban publikált adatok szerint EGF (epidermális növekedési faktor) ill. szérum fokozza a szérum-szegény médiummal szinkronizált normál NIH 3T3 sejtek osztódását. Ugyanakkor ez a hatás 60 %-osan gátlódott NIH M17

sejtekben dexametazon kezelés után, amikor az endogén Ras funkcióját a domináns gátló mutáns Ras fehérje (Ha-Ras Asn-17) termelôdésének indukálása csökkenti. Ezek a kísérleti eredmények azt igazolták, hogy a Ras fehérje mûködése szükséges az EGF ill. a szérum által megindított sejtosztódáshoz. Mivel aktív Rassal transzformált fibroblasztokban fokozódik a PC-PLC által katalizált foszfatidilkolin lebontás és az exogén PC-PLC enzim pedig normál fibroblasztokban mitogén hatású, felvetôdik a kérdés, hogy a PC-PLC enzim hatására kialakuló DNS szintézis és mitózis Ras-függô jelátviteli úttal közvetítôdik-e?

Ennek kiderítése céljából tríciummal jelölt timidin inkorporációját mértük a szövettenyészeti táptalajhoz adott különbözô mitózis induktorokkal való kezelés után. Kísérleteink szerint szérum, EGF, TPA és tisztított bakteriális PC-PLC enzim is fokozta az NIH 3T3 sejtek DNS-szintézisét. NIH M17 sejtekben viszont a dexametazon kezelés jelentôsen csökkentette a szérum hatását, majdnem megszüntette az EGF és a TPA okozta timidin beépülést, de nem volt hatással a PC-PLC által beindított DNS-szintézisre. Tehát az EGF, a TPA és a szérum hatása is Ras-függô, míg a PC-PLC enzim Rastól független.

A tisztított PC-PLC enzimmel kapott eredmények felvetették annak lehetôségét, hogy az észlelt hatásokért nem maga a PC-PLC enzim, hanem a preparátumot szennyezô anyagok a felelôsek. Ezért kolóniaképzôdés mérése során mutáns Rast ill. bakteriális PC-PLC-t expresszáló plazmidokkal különbözô kombinációkban transzfektáltunk normál NIH 3T3 sejteket. A mutáns Rast hordozó pZIP M17 plazmid hatására a sejtek szaporodása gátlódott, míg a mutáns Rast és a PC-PLC-t hordozó plazmidok együttes transzfekciója után a sejtszaporodás gátlását nem észleltük. Következésképpen a PC-PLC enzim termelôdése felfüggesztette a Ras funkció gátlásából eredô proliferáció csökkenést, és a szennyezôdés lehetôségét is kizártuk.

A fenti kísérleti eredmények két módon magyarázhatók: A PC-PLC által létrehozott változások vagy függetlenek a Ras mûködésétôl, vagy a Ras-függô jelátviteli mechanizmus részei, de magától a Ras fehérjétôl lefelé helyezkednek el ebben a láncolatban.

Igy a továbbiakban azt vizsgáltuk, hogy a domináns gátló Ras mutáns expressziója milyen hatással van az endogén PC-PLC által katalizált foszfatidilkolin hidrolízisre. A foszfatidilkolin lebontását kétféle enzim katalizálhatja a növekedési faktorok által kezelt sejtekben. Egyrészt a PC-PLC enzim aktivitása fokozódhat, ami diacilglicerint és foszfokolint képez. Másrészt a

PC-PLD is hozzájárulhat a metabolitok képzôdéséhez, aminek terméke kolin és foszfatidsav. Elôbbi a kolinkináz által foszfokolinná, utóbbi a foszfatidsav-foszfohidroláz által diacilglicerinné alakulhat. Az átfedések miatt a szóbajöhetô termékek képzôdésének kinetikáját vizsgáltuk.

Normál NIH 3T3 sejtekben a korai idôszakban (3-6 óra) a diacilglicerin képzôdése felülmúlta a foszfokolinét, ami arra utalt, hogy a diacilglicerin részben nem a PC-PLC enzim mûködésének következtében képzôdött. Ugyanakkor a késôbbiekben a két termék mennyisége párhuzamosan változott, alátámasztva azt a feltételezést, hogy ekkor már döntôvé vált a PC-PLC hatása. A kolin jelenlétének hiánya ugyancsak azt igazolta, hogy a PC-PLD részesedése elhanyagolható a diacilglicerin képzôdésben. NIH M17 sejtekben a dexametazonnal indukált mutáns Ras protein termelôdése kb. 50 %-ban csökkentette a szérum által okozott metabolit képzôdést és teljesen megszüntette az EGF hatását. Ugyanakkor a külsôleg a táptalajhoz adott PC-PLC legalább olyan intenzitással mûködött dexametazon jelenlétében, mint nélküle.

Ezért a metabolitok képzôdésének mérési adatai egyértelmûen bizonyították, hogy az endogén Ras mûködés csökkenése gátolta a szérum és az EGF által elôidézett foszfatidilkolin hidrolízist, következésképpen az NIH 3T3 sejtek mitogén növekedési faktorokkal való kezelését követôen a PC-PLC enzim Ras-függôen aktiválódott.

2. A PC-PLC enzim aktiválja a Raf-kinázt:

A korábbi kísérleti adatok és új megállapításaink arra utaltak, hogy mind a PC-PLC, mind a Raf kináz Ras-függô úton aktiválódnak. Kézenfekvô volt a kérdésfeltevés: Hogyan viszonyul egymáshoz az említett két Ras-effektor molekula a jelátviteli mechanizmusban? A kérdésre háromféle válasz adható: 1. A PC-PLC és a Raf kináz egymástól független utat képviselnek. 2. A PC-PLC a Raf aktivátora. 3. A Raf a PC-PLC aktivátora.

Mivel a kolóniaképzés mérésekor kotranszfekciót követôen a pZIP M17 plazmid sejtproliferációra gyakorolt gátló hatása kivédhetô volt pPC-PLC-vel, a domináns gátló Raf hatása viszont nem, az tûnik a legvalószínûbbnek, hogy a PC-PLC a Raf aktivátora.

Ezért a Raf foszforiláció mérésével azt próbáltuk meghatározni, hogy a PC-PLC enzim ténylegesen aktiválni képes-e a Raf kinázt. Irodalmi adatok

alapján ismert, hogy a Raf molekula aktiválódása során hiperfoszforilálódik, és a hiperfoszforilációs forma lassabban vándorol gélelektroforézis során, mint az eredeti molekula. Igy a hiperfoszforiláció jelensége a Raf aktivációjának mérésére használható. Normál NIH 3T3 sejtekben a PC-PLC, az EGF és a TPA is hiperfoszforilációt hozott létre, NIH M17 sejtekben azonban a mutáns ras génjének aktiválása gátolta az EGF és a TPA stimulálta Raf foszforilációt, de nem tudta gátolni a PC-PLC enzim hatását.

Ebbôl következett, hogy NIH 3T3 sejtekben az EGF, a TPA és a PC-PLC is fokozni tudta a Raf kináz aktivitását, de a felsorolt ágensek közül csak a PC-PLC hatása volt független a Rastól. Összességében a legvalószínûbb magyarázat szerint az EGF és a TPA aktiválták a Rast, majd ezen keresztül a PC-PLC molekulát és a Raf csak ezt követôen aktiválódott a jelátviteli mechanizmusban.

Feltevésünket a D609 (xantogenát triciklodekán-9-il) nevû gátlóanyag alkalmazásával tudtuk bizonyítani. Korábbi in vitro vizsgálatok és saját in vivo méréseink szerint a D609 specifikusan gátolja a PC-PLC enzim mûködését anélkül, hogy egyéb foszfolipázokra hatna. Kísérleteink szerint a TPA, az EGF és a szérum által elôidézett Raf hiperfoszforiláció nem jött létre akkor, ha a PC-PLC enzimet D609-cel gátoltuk, igy tehát TPA, EGF és szérum kezelés is a PC-PLC-n keresztül aktiválta a Raf kinázt.

Az elsô két pontban ismertetett kísérletek eredményei szerint az információ áramlás legvalószínûbb útját az alábbi séma szemlélteti:

Növekedési faktor receptorok

|

Ras

|

PC-PLC

|

Diacilglicerin képzôdés

|

Raf

|

MAP kináz kináz

|

MAP kináz

Fontos hozzátenni azonban, hogy a fenti lineáris útvonal helyett gyakran úgy tûnik, hogy elágazódó hálózattal állunk szemben, amelyben konvergenciák, divergenciák és visszacsatolási mechanizmusok is megjelennek.

A PC-PLC és a Raf kapcsolatára vonatkozóan egyelôre spekulációkra kell szorítkoznunk. Kétféle változat képzelhetô el. 1./ A diacilglicerin direkt Raf aktivátor. Ezt támasztaná alá, hogy a Raf amino terminális regulatórikus doménjében a PKC ligandum-kötôhelyéhez hasonló "zinc-finger" domén található, valamint az, hogy a PI-PLC eredetû diacilglicerin eltér a PC-PLC eredetûtôl. 2./ A Rafot a PKC, vagy annak egyik alfaja aktiválja diacilglicerin közbeiktatásával. Ezt valószínûsíti, hogy a PKC egyes izoenzimei foszforilálni és ezáltal aktiválni is képesek a Rafot. Mivel a PKC TPA elôkezeléssel való depletálása nem csökkenti a PC-PLC mitotikus hatását, a Rafot aktiváló PKC a nem-klasszikus, TPA elôkezelésre nem érzékeny izoenzimek közé kell hogy tartozzon.

Az ismertetett folyamatokat NIH 3T3 sejtekben bizonyítottuk, azonban PC12 sejtekben nem tudtuk igazolni. Mivel PC12 sejtekben a metabolit mérések eredményei nehezen magyarázhatók, az sem volt egyértelmûen eldönthetô, hogy az enzim mûködött-e ebben a sejttípusban. Az azonban nagyon valószínû, hogy az NGF indukálta differenciációban nincs jelentôs szerepe.

3. A PC12 sejtek differenciálódását kiváltó szinergizmusok:

Vizsgálatainkkal három fontos jelátviteli út (cAMP, protein kináz C és Ca^{++}) és a Ras protein kapcsolatát kívántuk meghatározni. Az adott jelátviteli utak közvetlen stimulálására másodlagos hírvivô analógokat használtunk, sorrendben dbcAMP-t, TPA-t és ionomicint. Ezen anyagok hatását hasonlítottuk össze normál PC12 sejtekben és olyan transzfektánsokban, amelyekben az endogén Ras protein mûködése gátolt volt. A másodlagos hírvivôket egyedül vagy NGF-fel együtt adagoltuk és a PC12 sejtek morfológiai differenciálódását mértük. Megfigyeléseink szerint egyedül adva egyik ágens sem okozott differenciációt a transzfektáns sejtekben, és a normál PC12 sejtekben is csak az NGF volt hatásos. Ezzel szemben NGF+dbcAMP vagy NGF+ionomicin együttes adásakor a transzfektált sejtek közepes mértékben differenciálódni tudtak (20-50 %). Hasonló hatást észleltünk dbcAMP+TPA esetén, míg az NGF+TPA kombináció nem változtatta meg a gátlást.

Mivel a transzfektáns sejtekben a Ras-függô utak gátoltak, így feltehetôen egy NGF indukálta, de Ras-független út játszik szerepet az ionomicinnel ill. cAMP-vel létrehozott szinergizmusban. Ez a Ras-független út nem lehet a cAMP vagy a Ca^{++} közvetítette út, mert azok maguk is a szinergizmus részesei. A protein kináz C volt egyik jelöltünk a Ras-független út szerepére, hiszen a TPA NGF-fel nem, de dbcAMP-vel szinergizmusban volt a transzfektáns sejtekben. A protein kináz C szerepének meghatározása céljából olyan sejtekben végeztünk differenciációs kísérleteket, amelyekben TPA elôkezeléssel depletáltuk a protein kináz C enzimet. A protein kináz C aktivitásának csökkenése nem befolyásolta az NGF+dbcAMP által okozott differenciációt, tehát maga a protein kináz C nem azonos az NGF-indukálta Ras-független úttal.

Ennek ellenére a TPA által stimulált korai gén válasz nem gátolt a Ha-ras Asn-17-tel transzfektált sejtekben, így lehetségesnek tûnt, hogy a protein kináz C felelôs a korai válasz gének NGF-indukciójáért a PC12 sejtek differenciációja során. A kérdés közelebbi vizsgálata céljából Northern hibridizációval mértük a c-fos indukcióját TPA elôkezelés után. TPA elôkezelés nem befolyásolta az NGF indukciót, vagyis a korai gének expressziójának indukálása nem protein kináz C-függô folyamat. A tranziens transzfekciók eredményei alapján a protein kináz C nem volt szükséges az NGF által kiváltott AP-1 promoter aktivációhoz sem.

Késôbbi MAP kináz méréseink során azt találtuk, hogy dbcAMP és NGF külön-külön alkalmazva nem aktivált a magas expressziójú transzfektáns sejtekben, azonban együttes használatuk esetén a MAP kináz mûködés fokozódása következett be. Mindez azt sugallja, hogy a dbcAMP-NGF szinergizmus hátterében a Raf-MAP kináz rendszer aktiválódása állhat.

4. A Raf-MAP kináz rendszer hosszú távú aktivációja nélkülözhetetlen a PC12 sejtek differenciálódása során:

Korábbi mérések alapján ismert volt, hogy a Raf-1 és a B-Raf protein kinázok, valamint a MAP kinázok aktiválása Ras-függô folyamat, és Ha-Ras Asn-17-tel stabilan transzfektált, NGF-fel kezelt sejtekben közel teljes mértékben gátolt. Sôt mi több, a Raf-1 kináz néhány sejttípusban aktiválni tudja a MAP kinázokat, és egy visszacsatolási mechanizmus is feltételezhetô.

Ezért az NGF kezelésre adott Raf ill. MAP kináz válasz változását mértük a Ras aktivitás mértékének függvényében azt kiderítendô, hogy mi áll az alacsony mutáns Ras expresszió által létrehozott differenciáció gátlás hátterében. A Raf proteinek (Raf-1 és B-Raf) aktivációjának méréséhez ismét a hiperfoszforiláció mérését használtuk, a MAP kináz aktivitást protein gélben in situ vizsgáltuk.

Eredményeink szerint a magas expressziójú sejtekben a Raf-1, a B-Raf és a MAP-kináz NGF által létrehozott stimulációja teljesen Ras-függônek tûnt. Valamivel alacsonyabb Ha-Ras Asn-17 expresszió esetén a Raf foszforiláció alig volt mérhetô és a MAP kináz aktivitás csak a maximális indukció 30%-át tette ki. Az alacsony expressziójú sejtekben többé-kevésbé ép Raf foszforiláció mellett jelentôs MAP kináz aktivitás csökkenés (egészen 50%-ig) volt azonosítható. Korábban közölt és jelen adataink összevetésével megállapítottuk, hogy a korai válasz gén aktiváció mértéke minden vizsgált sejtvonalban megegyezett a Raf-MAP kináz rendszer aktiválásának mértékével. Nem találtunk azonban magyarázatot arra vonatkozóan, hogy miért gátlódik a differenciáció már kisfokú Ras-gátlás esetében is. Ezt a jelenséget az eIF-4E transzlációs iniciációs faktor foszforilációjának mérésével sem tudtuk értelmezni.

EGF és NGF kezelés után mind a Raf, mind a MAP kináz aktiválódik a PC12 sejtekben, holott az EGF mitogén, az NGF pedig differenciációt okoz. Ugyanakkor az EGF általi aktiváció rövid idôtartamú az NGF elnyújtott hatásához viszonyítva. Ebbôl kiindulva azt feltételeztük, hogy az alacsony expressziójú sejtekben hasonló jelenség megy végbe. Feltételezésünk helyesnek bizonyult, hiszen méréseink szerint az említett transzfektáns sejtekben 30 perc után már csak a maximális aktivitás kb. 10%-a található meg, mig normál PC12 sejtekben ez az érték még több óra után is közel 50% lehet. Mindez azt jelenti, hogy az aktivitás korai lecsengése a differenciálódó képesség elvesztésének egyik tényezôje.

5. Rastól független utak szerepe a PC12 sejtek differenciációs folyamataiban:

A **PI-3-kináz** enzim foszfatidilinozitolt vagy foszfatidilinozitol-foszfátokat használ szubsztrátként és foszfatidilinozitol-3-foszfátokat képez. NGF hatására tirozinon foszforilálódik és aktiválódik, ami termékeinek felszaporodásával jár együtt. Aktivitása anti-foszfotirozin immunprecipitátumból meghatározható, de

anti-Ras antitesttel kicsapott fehérjék is rendelkeznek PI-3-kináz aktivitással. Ebbôl arra következtettünk, hogy a PI-3-kináz enzim a Rassal komplexet képez, és valószínûleg mûködése is Ras-függô.

Ennek igazolása érdekében domináns gátló Rassal stabilan transzfektált sejtekben mértük az anti-foszfotirozin immunprecipitátum PI-3-kináz aktivitását. Eredményeink szerint a foszfatidilinozitol-3-foszfát képzôdése nem gátlódott a transzfektáns sejtvonalakban, ami arra utal, hogy a PI-3-kináz nem Ras-effektor a PC12 sejtekben. Nyitott maradt a kérdés, hogy képes-e a Ras aktivitását szabályozni, azaz a Rastól felfelé szerepe van-e a jelátvitelben.

A feszültség-függô **Na+ csatornák** expressziója kulcsfolyamat a PC12 sejtek differenciációja során. Ezek a csatornák központi szerepet töltenek be az akciós potenciál létrehozásában és kritikusak az idegrendszeri információ továbbításban. Legfontosabb komponensük az alfa alegység, ami egymagában is mûködôképes csatornát tud kialakítani. Az alfa alegység család legalább négy gént foglal magába, ugymint I-es, II-es, IIa és III-as típus, amelyek expressziója eltérô az idegrendszer egyes részeiben. Ismeretes az is, hogy a Na^+ csatorna fehérjék termelôdése transzkripciós és poszttranszkripciós szinten is szabályozódhat.

Northern hibridizáció és RNáz védelem vizsgálat során azt tapasztaltuk, hogy NGF kezelés hatására a csatornák közül csak a II-es típusú fejezôdött ki a PC12 sejtekben. Ez az expresszió Rastól független úton történt és funkcióképes fehérjék kialakulásával járt együtt. Ebbôl következôen valószínûsíthetô, hogy a Ras-független késôi folyamatok szerepe már a közeljövôben hangsúlyozottabbá válik az NGF-indukálta differenciáció kutatásában.

Az a felismerésünk, hogy a Ha-Ras Asn-17 által elôidézett Ras blokk áthidalható NGF és ionomicin együttes alkalmazásával felvetette annak a lehetôségét, hogy a Ca^{++} által regulált utak a Rastól lefelé találhatók meg a jelátvitelben. Mivel a **foszfolipáz-C-gamma** többek között inozitol-trifoszfátot képez, ami a Ca^{++} sejten belüli mobilizációjáért felelôs, az inozitolfoszfát mennyiségének változását mértük NGF kezelés után. Normál PC12 sejtekben NGF hatására az inozitolfoszfát szint emelkedését tapasztaltuk, ami nem gátlódott egyik csökkent Ras aktivitású transzfektáns sejtben sem, így tehát magállapítottuk, hogy a foszfolipáz-C-gamma aktiválása a Ras proteintôl függetlenül mûködik a jelátvitel során.

6. A cAMP Ras/Raf/MAP kináz rendszerre gyakorolt hatásának összehasonlítása fibroblasztokban és PC12 sejtekben

Fibroblasztokban növekedési faktorok hatására aktiválódik a Ras-Raf-MAP kináz jelátviteli rendszer, ami végül az erre adott biológiai válaszban, a sejtosztódásban jut érvényre. Ezen sejtekben a cAMP kezelés a Raf kináz gátlásán keresztül csökkenti a MAP kináz aktiválást és következményesen a sejtosztódás ütemét is. Ezzel ellentétben PC12 sejtekben a cAMP nem gátolja a Ras aktiválását követô biológiai választ, azaz a neuronális differenciációt. Ezért elôször arra kerestük a választ, hogy mi történik a MAP kináz aktivitással PC12 sejtek cAMP kezelésének hatására.

Gélen belüli in situ MAP kináz méréssel azt találtuk, hogy a cAMP kezelés önmagában is aktiválja a MAP kináz enzimet. Ez csak PC12 sejtekben figyelhetô meg, fibroblasztokban ilyen változás nem észlelhetô. Növekedési faktorok és cAMP együttes adásakor viszont gátlás helyett a MAP kináz aktivitásának szinergisztikus növekedése a válasz. Ez a szinergizmus a neuronális differenciáció során is megfigyelhetô volt.

Korábban leírt adatok szerint fibroblasztokban a Raf-1 izoenzim aktiválja a MAP kinázt és ennek müködése gátlódik a cAMP aktiválta protein kináz A általi foszforiláláció után. Mivel PC12 sejtekben a Raf-1 mellett egy másik izoenzim, a Raf-B is nagy mennyiségben található, ezért azt feltételeztük, hogy a Raf-B izoenzim nem érzékeny a protein kináz A általi foszforilációra és gátlásra. Feltételzésünket alátámasztotta az a tény, hogy a protein kináz A enzim konszenzus felismerôhelye jelen van a Raf-1 molekulában, azonban hiányzik a Raf-B-bôl. Méréseink során azt találtuk, hogy a szérummentes környezetben tartott sejtekben cAMP kezeléssel mindkét Raf gátolható. Viszont ha a sejteket szérum jelenlétében növesztettük, akkor a B-Raf molekula sokkal kevésbé volt érzékeny a gátlásra. Ez arra utalt, hogy a B-Raf és a Raf-1 cAMP iránti érzékenysége eltérô, nevezetesen a B-Raf sokkal nehezebben gátolható. Ez a megfigyelés annak a lehetôségét vetette fel, hogy a B-Raf kifejezôdése hozzájárul a sejtspecifikus különbségekhez.

Ennek a kérdésnek a megválaszolása érdekében B-Rafot expresszáló plazmiddal stabilan transzfektáltunk Rat-1 fibroblasztokat. Az így létrehozott B-Rafot kifejezô sejteket a normál, B-Raf nélküli sejtekkel hasonlítottuk össze. Eredményeink szerint a fibroblasztokban kifejezôdô B-Raf ugyanúgy csak kissé érzékeny a cAMP gátlásra, mint PC12 sejtekben. Ennek hatására a MAP kináz

gátlása is kisebb, habár a PC12 sejtekben található szinergizmus nem alakul ki. Ez utóbbit arra vezettük vissza, hogy a két sejttípus nemcsak a B-Raf kifejezôdésében tért el egymástól, hanem abban is, hogy a cAMP önmagában csak PC12 sejtekben volt képes aktiválni a MAP kinázt. Ez a hatás a B-Rafot kifejezô transzfektált fibroblasztokban sem alakult ki. A fentiekkel összhangban volt, hogy a B-Rafot kifejezô fibroblasztok növekedési sajátságai is megváltoztak. A B-Raf bejuttatása csökkentette ill. megszüntette a cAMP növekedésre gyakorolt gátló hatását.

Külön szeretnék megemlékezni arról a megfigyelésünkrôl, hogy a cAMP kezelés Ras-függô módon aktiválta a MAP kinázt PC12 sejtekben. Ez a sejtspecifikus hatás amellett szól, hogy a cAMP rendszer nemcsak a Rastól lefelé elhelyezkedô Raf kinázokon keresztül kommunikál a tirozinkináz által kiváltott jelátviteli utakkal, hanem a Rastól felfelé is találunk interakciós pontot. Jelenlegi vizsgálataim arra irányulnak, hogy kiderítsem, mi ez a Rastól felfelé elhelyezkedô találkozási pont.

7. Az új eredmények összefoglalása:

Az ismertetett munka során a Ras protein jelátvitelben betöltött szerepének vizsgálata állt a középpontban. A bemutatott kísérletek két fô csoportra oszthatók. Egyrészt kiemelten foglalkoztam a PC-PLC enzim, mint Ras-függô út vizsgálatával és mitózisban betöltött szerepével. Másrészt NGF által indukált, Rastól függô, vagy tôle független utak PC12 sejtek differenciációjára gyakorolt hatását elemeztem. Az utóbbi kísérletekben fokozott hangsúlyt kapott a cAMP hirvivô rendszer modifikáló hatásának leírása.

1./ A tézisek anyagának része az a kísérletsorozat, amelyben elôször bizonyítottuk direkt módon **a PC-PLC Ras-effektor** szerepét.

2./ Megállapítottuk, hogy **a PC-PLC által képzett másodlagos hírvivôk aktiválják a Raf kinázt**.

3./ Felismertük, hogy **az NGF és dbcAMP vagy az NGF és ionomicin kezelés áthidalja a domináns gátló Ras differenciációra kifejtett hatását** a mutáns Rast expresszáló sejtekben. Ez a szinergizmus további lehetôségeket tárt fel az NGF indukálta, Ras-független jelek azonosításában.

4./ Kiderítettük, hogy **a Raf-MAP kináz rendszer hosszú távú gátlása felelôs a mutáns Rast alacsony szinten expresszáló PC12 sejtekben a differenciáció gátlásáért.**

5./ Kimutattuk, hogy **a PI-PLC, a PI-3-kináz és a Na^+ csatorna fehérjék NGF kezelés hatására Ras-független úton aktiválódnak**.

6./ Bebizonyítottuk, hogy a cAMP által aktivált **protein kináz A szelektíven befolyásolja a Raf izoenzimek müködését**. Ez magyarázza a cAMP fibroblasztok ill. PC12 sejtek növekedésére kifejtett ellentétes hatását.

7./ Azonosítottunk egy új lehetséges jelátviteli utat, nevezetesen a **cAMP által Ras függôen aktivált MAP kináz**t. Ez az elsô direkt bizonyítéka lehet annak, hogy a cAMP a Rastól felfelé is interakcióban van a tirozinkináz függô jelekkel.

Rövidítésjegyzék:

AP-1 = aktiváló protein-1 (promoter elem)
cAMP = ciklikus AMP
c-fos = celluláris fos onkogén
D609 = xantogenát triciklodekán-9-il (PC-PLC gátló)
dbcAMP = dibutiril cAMP
EGF = epidermális növekedési faktor
eIF-4E = eukarióta iniciációs faktor 4E
MAP kináz = mitogének aktiválta protein kináz
NIH 3T3 = egér fibroblaszt sejtvonal
NIH M17 = domináns negativ Rast expresszáló NIH 3T3 sejtvonal
NGF = idegsejt növekedési faktor
PC12 = patkány feokromocitóma sejtvonal
PC-PLC = foszfatidilkolin specifikus foszfolipáz C
PC-PLD = foszfatidilkolin specifikus foszfolipáz D
PI-3-kináz = foszfatidilinozitol-3-kináz
PI-PLC = foszfatidilinozitol specifikus foszfolipáz C
PKC = protein kináz C
pPC-PLC = PC-PLC-t expresszáló plazmid

pZIP M17 = domináns negativ Rast expresszáló plazmid
RNáz = ribonukleáz
TPA = forbol észter

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Hydrolysis of Phosphatidylcholine Is Stimulated by Ras Proteins during Mitogenic Signal Transduction

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We have used a dominant inhibitory *ras* mutant (Ha-*ras* Asn-17) to investigate the relationship of Ras proteins to hydrolysis of phosphatidylcholine (PC) in the transduction of mitogenic signals. Expression of Ha-Ras Asn-17 inhibited NIH 3T3 cell proliferation induced by polypeptide growth factors or phorbol esters. In contrast, the mitogenic activity of PC-specific phospholipase C (PC-PLC) was not inhibited by Ha-Ras Asn-17 expression. Similarly, cotransfection with a cloned PC-PLC gene bypassed the block to NIH 3T3 cell proliferation resulting from expression of the inhibitory *ras* mutant. Hydrolysis of PC can therefore induce cell proliferation in the absence of normal Ras activity, suggesting that PC-derived second messengers may act downstream of Ras in mitogenic signal transduction. This was substantiated by the finding that Ha-Ras Asn-17 expression inhibited growth factor-stimulated hydrolysis of PC. Taken together, these results indicate that PC hydrolysis is a target of Ras during the transduction of growth factor-initiated mitogenic signals.

The *ras* proto-oncogenes encode plasma membrane-associated guanine nucleotide binding proteins that function in signal transduction pathways leading to cell proliferation in response to mitogenic growth factors (4, 9, 10, 23, 34) and to neuronal differentiation induced by nerve growth factor (1, 12, 25, 35). The Ras proteins are associated with protein-tyrosine kinase growth factor receptors via GTPase activating protein (GAP), which regulates Ras activity and presumably serves to couple Ras to growth factor stimulation (15, 16, 22). In yeasts, Ras proteins act to regulate adenylate cyclase (37). However, despite the importance of *ras* oncogenes and proto-oncogenes in both neoplastic and normal cell growth, the targets of Ras in mammalian cells have not been identified.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate is stimulated by a variety of growth factors, yielding diacylglycerol and inositol triphosphate, which activate protein kinase C and mobilize intracellular calcium, respectively (2, 24). In addition, a number of growth factors have been found to stimulate hydrolysis of a second phospholipid, phosphatidylcholine (PC), generating diacylglycerol and phosphocholine (8). PC hydrolysis also appears to be stimulated in cells expressing oncogenic Ras proteins (17, 21, 30, 39). Moreover, treatment of quiescent cells with exogenous PC-specific phospholipase C (PC-PLC) is itself mitogenic (18). Importantly, PC-PLC induces cell proliferation even if protein kinase C is downregulated by pretreatment with phorbol esters (18). It thus appears that PC hydrolysis may generate a novel second messenger, possibly phosphocholine or a species of diacylglycerol that acts on a target other than phorbol ester-sensitive members of the protein kinase C family.

We have used a dominant inhibitory *ras* mutant (9) to investigate the role of Ras in signal transduction pathways in mammalian cells. The protein encoded by this mutant *ras* gene (Ha-*ras* Asn-17) preferentially binds GDP versus GTP (9) and appears to specifically interfere with the function of endogenous proto-oncogene Ras proteins (33). Expression of Ha-Ras Asn-17 inhibits the proliferation of NIH 3T3 cells and the mitogenic response of these cells to a variety of polypeptide growth factors as well as to direct activation of protein kinase C by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4). In contrast to its activity in NIH 3T3 cells, Ha-Ras Asn-17 does not affect proliferation of PC12 pheochromocytoma cells but blocks the neuronal differentiation of these cells induced by nerve growth factor (35).

We report here that PC-PLC can bypass the block to proliferation of NIH 3T3 cells resulting from Ha-Ras Asn-17 expression and, conversely, that expression of Ha-Ras Asn-17 in NIH 3T3 cells inhibits growth factor-stimulated hydrolysis of PC. Taken together, these findings indicate that PC-derived second messengers function downstream of Ras in mitogenic signal transduction.

MATERIALS AND METHODS

Cell lines. NIH(M17) cells are a transfected subclone of NIH 3T3 cells in which the Ha-*ras* Asn-17 gene is expressed from the dexamethasone-inducible mouse mammary tumor virus long terminal repeat (4). NIH 3T3 and NIH(M17) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum. The stably transfected NIH(M17) cell line was maintained in medium containing G418 (400 μg/ml).

PC-PLC. PC-PLC was purified from *Bacillus cereus* as previously described (18).

Plasmid DNAs. The *ras* expression plasmid pZIP M17 contains the Ha-*ras* Asn-17 gene inserted into the *Bam*HI site of pZIPneoSV(X) so that the mutant *ras* gene is coexpressed with *neo* (9). The PC-PLC expression plasmid

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TABLE 1. Mitogenic activity of PC-PLC

Mitogen	DNA synthesis (fold induction)[a]					
	NIH 3T3 cells			NIH(M17) cells		
	−Dex	+Dex	Ratio[b]	−Dex	+Dex	Ratio
Serum	16 ± 1	19 ± 2	1.2	17 ± 1	6 ± 1	0.4
EGF	9 ± 0.7	9 ± 0.8	1.0	10 ± 2	1 ± 0.1	0.1
TPA	10 ± 1	8 ± 0.4	0.8	9 ± 2	1 ± 0.1	0.1
PC-PLC	15 ± 0.3	15 ± 0.3	1.0	15 ± 1	15 ± 0.3	1.0

[a] NIH 3T3 or NIH(M17) cells were incubated 24 h in media containing 0.5% calf serum and dexamethasone (Dex; 5×10^{-7} M) where indicated to induce quiescence. Cells were then stimulated by addition of calf serum (10%), EGF (10 ng/ml), TPA (100 nM), or PC-PLC (0.5 U/ml). [^{3}H]thymidine incorporation was determined 16 h after mitogenic stimulation. Data are presented as the ratio of [^{3}H]thymidine incorporation in stimulated cultures to that in unstimulated controls. Results are the averages ± standard errors of the mean of two or three independent assays, each containing duplicate plates.

[b] Ratio of stimulation of DNA synthesis with dexamethasone to that without dexamethasone.

pOPLCneo$^-$ was constructed from the *B. cereus* PC-PLC gene (14). The coding region for the 24-amino-acid signal peptide and the 14-amino-acid propeptide of *B. cereus* PC-PLC was replaced by the 21-amino-acid signal peptide of the *Escherichia coli* outer membrane protein OmpA. This construct, including 19 bp upstream of the start codon, was then inserted into the mammalian expression vector pMAMneo, and then the *neo* gene was deleted. Mammalian cells transfected with this plasmid display an increased level and intracellular activity of PC-PLC with no detectable secretion of the enzyme.

Transfection assays. NIH 3T3 cells were transfected with plasmid DNAs in the presence of 20 μg of carrier calf thymus DNA (Sigma Chemical Co.) as described previously (9). Cells were subcultured into medium containing G418 (400 μg/ml) 3 days after transfection, and neomycin-resistant colonies were either stained and counted or isolated for further study.

Mitogen-stimulated DNA synthesis. NIH 3T3 or NIH(M17) cells were plated in medium containing 10% calf serum at a density of 10^5 cells per 60-mm dish. The following day, media were changed to 0.5% calf serum, and dexamethasone (5×10^{-7} M) was added where indicated. Cells were incubated for 24 h to induce quiescence and then stimulated by the addition of serum or growth factors or by treatment with PC-PLC. DNA synthesis was assayed 16 h after mitogenic stimulation by labeling with [^{3}H]thymidine as described previously (4).

Northern (RNA) blot analysis. Cytoplasmic RNAs were isolated, electrophoresed in 1% agarose-formaldehyde gels, and hybridized with ^{32}P-labeled human Ha-*ras* probe as previously described (4).

Western blot (immunoblot) analysis. Protein samples (50 μg) were electrophoresed in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, transferred to nitrocellulose filters, and analyzed with anti-Ras antibody Ras-10 (New England Nuclear). Blots were developed by using goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate.

Growth factor-stimulated PC hydrolysis. NIH 3T3 or NIH(M17) cells (10^6 cells per 60-mm plate) were labeled for 48 h with [^{14}C]choline (2 μCi per culture, 50 to 60 mCi/mmol; New England Nuclear) or [^{3}H]myristic acid (10 μCi per culture, 30 Ci/mmol; New England Nuclear). The last 24 h of labeling was performed in media containing 0.5% calf serum and 5×10^{-7} M dexamethasone (where indicated). Cells were then washed and treated with serum, growth factors, or PC-PLC. Phosphocholine and diacylglycerol were extracted and analyzed by thin-layer chromatography as previously described (32). Radioactivity present as [^{14}C]phosphocholine was quantitated by β-scanning. [^{3}H]diacylglycerol was quantitated by excising and counting the appropriate regions of the thin-layer chromatography plates in a liquid scintillation counter.

RESULTS

Expression of Ha-Ras Asn-17 does not inhibit mitogenesis induced by PC-PLC. We initially investigated the effect of Ha-Ras Asn-17 expression on the mitogenic activity of exogenous PC-PLC. NIH(M17) cells are a transfected subclone of NIH 3T3 cells in which the Ha-*ras* Asn-17 gene is expressed via the mouse mammary tumor virus promoter. These cells proliferate normally in the absence of dexamethasone, but their proliferation is reversibly inhibited by induction of the mutant *ras* gene (4). As previously reported (4), the mitogenic response of NIH(M17) cells to serum was inhibited approximately 60% by dexamethasone induction of Ha-Ras Asn-17, and the mitogenic response to epidermal growth factor (EGF) or TPA was virtually abolished by Ha-Ras Asn-17 induction (Table 1).

Addition of PC-PLC purified from *B. cereus* to the culture medium of NIH 3T3 cells was strongly mitogenic, consistent with previous results (18). Importantly, dexamethasone induction of Ha-Ras Asn-17 did not inhibit the mitogenic response of NIH(M17) cells to PC-PLC (Table 1). Thus, in contrast to serum, growth factors, and TPA, the mitogenic activity of PC-PLC did not appear to be dependent upon Ras activity, suggesting that PC-derived second messengers might function downstream of Ras in mitogenic signal transduction.

Cotransfection with a cloned PC-PLC gene overcomes Ha-Ras Asn-17 inhibition of NIH 3T3 cell proliferation. In order to ensure that the mitogenic activity of PC-PLC was not due to potential contaminants in the enzyme preparation, we tested the ability of a cloned PC-PLC gene to overcome the growth inhibitory activity of Ha-Ras Asn-17. NIH 3T3 cells were transfected with a neomycin resistance gene either alone [the pZIPneoSV(X) vector] or linked to Ha-*ras* Asn-17 (the pZIP M17 plasmid). Results of a representative experiment are illustrated in Fig. 1 and data from three independent experiments are presented in Table 2.

As previously observed (9), Ha-*ras* Asn-17 inhibited the proliferation of transfected cells, greatly reducing the number of neomycin-resistant transformants obtained with pZIP M17 compared with those obtained with the pZIPneoSV(X) vector containing *neo* alone. Cotransfection with a PC-PLC expression plasmid reversed the inhibitory effect of the

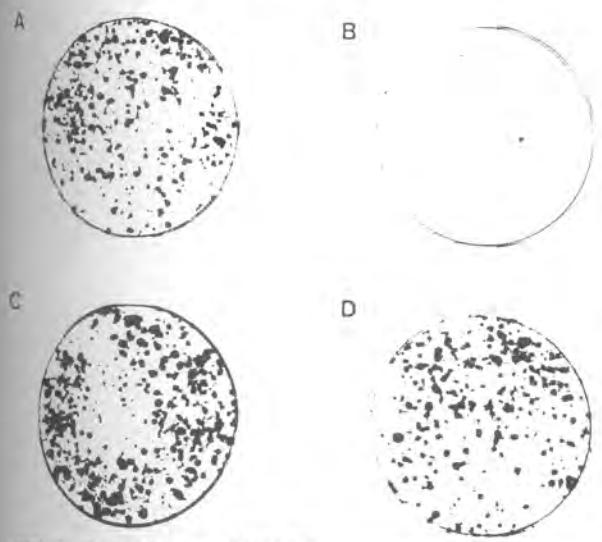

FIG. 1. Cotransfection of NIH 3T3 cells with Ha-*ras* Asn-17 and a PC-PLC expression plasmid. NIH 3T3 cells were transfected with plasmid DNAs and subcultured into medium containing G418. Colonies were stained and photographed 17 days after transfection. (A) pZIPneoSV(X) (0.1 μg); (B) pZIP M17 (0.1 μg); (C) pZIP-neoSV(X) (0.1 μg) plus pOPLCneo$^-$ (0.5 μg); (D) pZIP M17 (0.1 μg) plus pOPLCneo$^-$ (0.5 μg).

mutant *ras* gene, so that the yield of neomycin-resistant transformants obtained by cotransfection with the pZIP M17 plasmid plus PC-PLC was similar to that obtained with the pZIPneoSV(X) vector containing *neo* alone (Fig. 1 and Table 2). These results are similar to those obtained after cotransfection of pZIP M17 together with plasmids expressing *raf* oncogenes (9) and confirm that PC-PLC can stimulate cell proliferation independently of normal Ras activity.

The ability of PC-PLC to relieve the growth inhibitory effect of Ha-Ras Asn-17 was further verified by analysis of expression of the mutant *ras* gene in the neomycin-resistant transformants obtained in the cotransfection assays. Expression of the transfected Ha-*ras* Asn-17 gene was detected by Northern and Western blot analysis of five neomycin-resistant cell lines obtained after cotransfection with the pZIP M17 plasmid plus PC-PLC. Each of these cell lines expressed 5.6-kb Ha-*ras* transcripts (Fig. 2A, lanes 1 to 5), as expected for expression of the mutant *ras* gene in the ZIP vector (9). In contrast, no expression of the transfected Ha-*ras* Asn-17 gene was detected in five neomycin-resistant cell lines obtained after transfection with the pZIP M17 plasmid alone (Fig. 2A, lanes 6 to 10), indicating that these rare neomycin-resistant transformants did not express detectable levels of the inhibitory *ras* mutant. Consistent with the RNA results, the level of Ras protein was increased in the five cell lines that were cotransfected with pZIP M17 plus PC-PLC (Fig. 2B, lanes 1 to 5), whereas cells transfected with pZIP M17 alone expressed low levels of Ras protein similar to that in NIH 3T3 cells (Fig. 2B, lanes 6 to 10). Expression of the Ha-*ras* Asn-17 gene in cells that were cotransfected with PC-PLC therefore indicates that PC-PLC is able to overcome the inhibitory effect of Ha-Ras Asn-17 on cell proliferation.

FIG. 2. Expression of Ha-*ras* Asn-17 in NIH 3T3 cells cotransfected with PC-PLC. (A) Cytoplasmic RNA (15 μg per lane) was electrophoresed in 1% agarose-formaldehyde gels and analyzed by Northern blot hybridization with ^{32}P-labeled human Ha-*ras* probe. Lanes 1 to 5, five independent lines of G418-resistant NIH 3T3 cells cotransfected with pZIP M17 plus pOPLCneo$^-$; lanes 6 to 10, G418-resistant NIH 3T3 cells isolated after transfection with pZIP M17 alone. Each lane contained similar amounts of 18S and 28S rRNAs. (B) Cell extracts (50 μg of protein) were electrophoresed in an SDS–12% polyacrylamide gel and analyzed by immunoblotting with anti-Ras antibody Ras-10. The position of the Ras protein (p21) is indicated. The lanes are as described for panel A.

TABLE 2. Cotransfection of PC-PLC with Ha-*ras* Asn-17

Expt	No. of neomycin-resistant colonies[a]			
	ZIP	ZIP + PC-PLC	pZIP M17	pZIP M17 + PC-PLC
1	340 ± 20	328 ± 23	23 ± 3	290 ± 4
2	252 ± 13	257 ± 19	13 ± 2	238 ± 12
3	222 ± 12	234 ± 19	14 ± 4	217 ± 16

[a] NIH 3T3 cells were transfected with 0.1 μg of pZIPneoSV(X) (designated ZIP) or 0.1 μg of pZIP M17. Where indicated, cultures were cotransfected with 0.5 μg of a PC-PLC expression plasmid (pOPLCneo$^-$). Cells were subcultured into medium containing G418. 3 days after transfection, and neomycin-resistant colonies were stained and counted 2 weeks later. Results of three independent experiments are shown. Data are the averages of duplicate or triplicate plates ± standard errors of the mean.

Ha-Ras Asn-17 expression inhibits PC hydrolysis in response to mitogenic growth factors. The ability of PC-PLC to bypass the Ha-Ras Asn-17 block to cell proliferation indicates that PC-PLC acts either downstream or independently of Ras in mitogenic signal transduction. To distinguish between these possibilities, we investigated the effect of Ha-Ras Asn-17 expression on the growth factor-stimulated hydrolysis of PC.

Hydrolysis of PC in response to growth factors can be catalyzed by either PC-PLC or PC-PLD (8). The former yields phosphocholine plus diacylglycerol, while the latter yields choline plus phosphatidic acid. Since choline can be subsequently converted to phosphocholine by choline kinase and phosphatidic acid to diacylglycerol by phosphatidate phosphohydrolase, the actions of PC-PLC and PC-PLD can

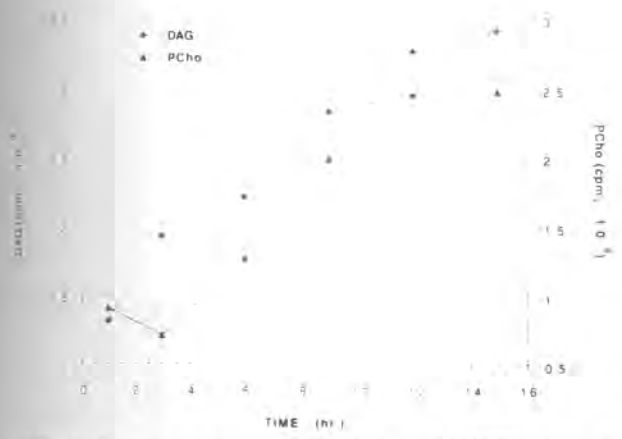

FIG. 3. Serum stimulation of PC hydrolysis. NIH 3T3 cells were labeled with [^{14}C]choline or [^{3}H]myristic acid for 48 h with the last 24 h of labeling performed in medium containing 0.5% calf serum. Cells were then washed and stimulated by addition of 10% calf serum. The production of [^{14}C]phosphocholine (PCho) and [^{3}H]diacylglycerol (DAG) was determined at the indicated times. Data are the averages of duplicate plates for phosphocholine and triplicate plates for diacylglycerol.

yield similar products. In order to characterize PC turnover, we therefore investigated the kinetics of production of choline, phosphocholine, diacylglycerol, and phosphatidic acid in response to mitogenic stimulation of NIH 3T3 cells.

Cells were prelabeled with either [^{14}C]choline or [^{3}H]myristic acid, which is incorporated specifically into PC (13, 32), and then stimulated by addition of serum. Elevated levels of both phosphocholine and diacylglycerol were detectable within 6 h after serum addition and continued to increase for at least 12 h thereafter (Fig. 3). An increase in diacylglycerol was apparent somewhat earlier than phosphocholine (3 h after serum addition), which might suggest production of [^{3}H]myristate-labeled diacylglycerol from another source at early times after mitogen stimulation. The parallel production of both diacylglycerol and phosphocholine for 6 to 12 h after serum addition is similar to previous results (18) and indicates that PC hydrolysis is a long-term response to mitogenic stimulation.

In contrast to the serum-stimulated production of [^{14}C]phosphocholine, no [^{14}C]choline was detected in either untreated or serum-stimulated cells (Fig. 4). The production of phosphocholine in the absence of any detectable choline suggests that the serum-stimulated hydrolysis of PC was catalyzed by PC-PLC rather than PC-PLD, although the possibility that choline was rapidly converted to phosphocholine by the action of choline kinase cannot be excluded.

Phosphatidic acid, as well as diacylglycerol, was produced after serum stimulation of [^{3}H]myristic acid-labeled cells (data not shown). We therefore further investigated the possibility that diacylglycerol was being generated via a PC-PLD pathway by addition of 250 μM propanolol, an established inhibitor of phosphatidate phosphohydrolase (3), at the time of serum stimulation. In contrast to its inhibition of diacylglycerol production resulting from the action of PC-PLD in other systems (3, 21, 32), propanolol had no effect on the amount of diacylglycerol detected 14 h after serum addition (data not shown). It thus appears that the action of PC-PLC, rather than PC-PLD, was primarily responsible for generation of both diacylglycerol and phosphocholine in serum-stimulated NIH 3T3 cells.

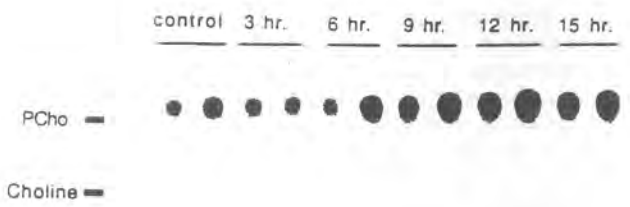

FIG. 4. Formation of phosphocholine from serum-stimulated PC hydrolysis. NIH 3T3 cells were labeled with [^{14}C]choline, made quiescent, washed, and stimulated with 10% calf serum as described in the legend to Fig. 3. Choline and phosphocholine (PCho) were extracted and analyzed by thin-layer chromatography and then by autoradiography. The results obtained with duplicate cell cultures are shown. The positions of choline and phosphocholine markers are indicated.

The effect of Ha-Ras Asn-17 expression on mitogen-stimulated PC hydrolysis, assayed by production of both diacylglycerol and phosphocholine, was then investigated (Fig. 5). Both serum and EGF induced PC turnover in NIH 3T3 cells and in NIH(M17) cells in the absence of dexamethasone. Expression of Ha-Ras Asn-17, however, inhibited serum stimulation of PC turnover by about 50% and completely blocked the stimulatory effect of EGF. These inhibitory effects of Ha-Ras Asn-17 on PC turnover are parallel to the effects of the mutant *ras* gene on serum and EGF-induced mitogenesis (compare with Table 1). In contrast, expression of Ha-Ras Asn-17 did not inhibit PC hydrolysis induced by treatment with exogenous PC-PLC. As previously reported (18), addition of the bacterial enzyme to the culture medium stimulated the production of intracellular phosphocholine as well as diacylglycerol, indicating uptake of the enzyme by the cells. It thus appeared that the inhibitory Ras mutant specifically interfered with serum and EGF-stimulated PC hydrolysis, indicating that normal Ras function is required for stimulation of PC turnover in response to mitogenic growth factors in NIH 3T3 cells.

DISCUSSION

Previous studies have implicated PC hydrolysis as a source of second messengers in response to stimulation by growth factors and oncogenic Ras proteins (8, 17, 18, 21, 39). In the present experiments, we have used a dominant inhibitory *ras* mutant to directly probe the relationship between PC hydrolysis and Ras in mitogenic signal transduction. In NIH 3T3 cells, treatment with exogenous PC-PLC or transfection with a cloned PC-PLC gene was found to bypass the block to proliferation resulting from expression of Ha-Ras Asn-17, indicating that PC-derived second messengers acted either downstream or independently of Ras to signal cell division. In addition, hydrolysis of PC in response to stimulation of NIH 3T3 cells by serum and EGF was inhibited by expression of the mutant *ras* gene. Moreover, the extent to which Ha-Ras Asn-17 inhibited PC hydrolysis stimulated by serum and EGF paralleled its effectiveness in inhibiting mitogenesis induced by these agents. Taken together, these results implicate PC hydrolysis as an important target of Ras in mitogenic signal transduction.

Hydrolysis of PC in response to growth factors and oncogenes can be catalyzed by either PC-PLC or PC-PLD, yielding diacylglycerol and phosphocholine or phosphatidic

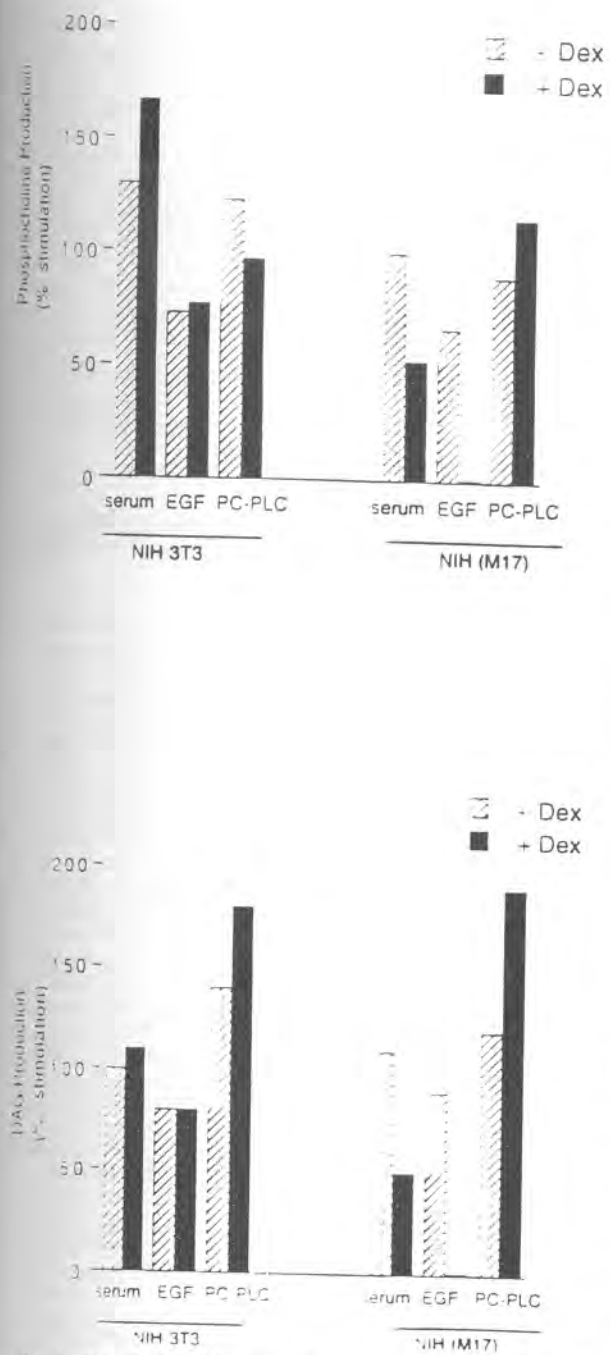

FIG. 8. The effect of Ha-Ras Asn-17 on mitogen-stimulated PC hydrolysis. NIH 3T3 or NIH(M17) cells were labeled for 48 h with [^{14}C]choline (upper panel) or [^{3}H]myristic acid (lower panel). The last 24 h of labeling was performed in medium containing 0.5% calf serum and 5×10^{-7} M dexamethasone (Dex) where indicated. Cells were then washed and treated with calf serum (10%), EGF (10 ng/ml), or PC-PLC (0.5 U/ml) for 14 h, and the amount of [^{14}C]phosphocholine or [^{3}H]diacylglycerol was determined. Data represent the averages of duplicate or triplicate plates and are presented as the percent stimulation of PC hydrolysis compared with that in untreated control cultures.

acid and choline, respectively (8). Phosphatidic acid can subsequently be converted to diacylglycerol by phosphatidate phosphohydrolase, and choline can be converted to phosphocholine by choline kinase. Previous studies have indicated that PC-PLC, rather than PC-PLD, is the primary catalyst of PC turnover in 3T3 cells stimulated by either platelet-derived growth factor or *ras* oncogene expression (18, 21). PC-PLD, however, also appears to be stimulated in 3T3 cells in response to platelet-derived growth factor or expression of the *src* oncogene (29, 32), potentially as a result of the activation of PC-PLD by protein kinase C (5). In the present study, hydrolysis of PC resulted in the formation of phosphocholine in the absence of any detectable choline production, suggesting that Ras-mediated PC turnover resulted primarily from the action of PC-PLC. In addition, the generation of diacylglycerol was not inhibited by propanolol, an inhibitor of phosphatidate phosphohydrolase. It thus appears that PC-PLC, rather than PC-PLD, is primarily responsible for Ras-mediated PC turnover.

In light of these findings, the consequences of unregulated expression of PC-PLC on cell growth are of interest. Cells transfected with the PC-PLC expression plasmid have a higher content of both phosphocholine and diacylglycerol as well as a detectable level of intracellular *B. cereus* PC-PLC, as observed in immunostaining experiments with an affinity-purified anti-PC-PLC polyclonal antibody (22a). In addition, expression of the *B. cereus* PC-PLC gene in NIH 3T3 cells results in a reduced serum dependence as well as the ability to form colonies in soft agar (22a). Chronic stimulation of PC hydrolysis thus appears to induce at least some properties characteristic of cell transformation.

The targets of PC-derived second messengers remain to be determined. As expected, since diacylglycerol is a product, some effects of PC hydrolysis are mediated by protein kinase C and can be blocked by downregulation of protein kinase C resulting from prolonged treatment with phorbol esters, such as TPA (6). However, both Ras and PC-PLC also appear to activate signal transduction pathways that are not blocked by phorbol ester downregulation of protein kinase C (4, 7, 18, 20). One possibility is that phosphocholine acts as a second messenger, but this seems unlikely since phosphocholine is a major cellular metabolite. The more likely candidate for a PC-derived second messenger therefore appears to be diacylglycerol. In this regard, it is noteworthy that distinct molecular species of diacylglycerol are generated by hydrolysis of PC and phosphatidylinositol 4,5-bisphosphate, so PC-derived diacylglycerols might be associated with novel second messenger activities (8, 11, 19, 28). Moreover, protein kinase C denotes a family of isozymes consisting of at least seven members, which may differ in both catalytic and regulatory properties (24, 26). In particular, the ζ isozyme of protein kinase C does not bind phorbol esters (27) and is therefore insensitive to downregulation by treatment with TPA. It is therefore possible that activation of specific isozymes of protein kinase C by PC-derived diacylglycerol is involved in the Ras and PC-PLC signaling events that are not blocked by TPA downregulation.

Alternatively, PC-derived diacylglycerols might activate other protein kinases. In this regard, recent studies have shown that expression of Ha-Ras Asn-17 blocks the activation of MAP kinase (36, 40) and the Raf proto-oncogene kinase (38, 40) in PC12 cells, as well as the activation of Raf in NIH 3T3 cells (38). In addition, Raf is able to bypass the inhibition of both NIH 3T3 cell proliferation (9, 31) and PC12 neuronal differentiation (38) resulting from interference with Ras proto-oncogene function. The Raf and MAP kinases therefore appear to act downstream of Ras in signal transduction pathways, raising the possibility that their activities are regulated by a PC-derived second messenger. However, it is also possible that stimulation of PC turnover and

activation of these kinases are distinct, independent targets of Ras. Indeed, previous studies indicated that Ras is coupled to at least two distinct signaling pathways in both the NIH 3T3 and PC12 cell systems (4, 35).

Finally, it is noteworthy that the turnover of PC induced by mitogens is a long-term response, persisting for several hours after growth factor treatment, and may thus provide a source of diacylglycerol leading to prolonged activation of protein kinase C or other targets (8, 18). Ras activity is similarly thought to be required for several hours following mitogen stimulation, since microinjection of anti-Ras antibodies 6 to 8 h after growth factor exposure still inhibits the initiation of DNA synthesis (23). The persistence of PC-derived second messengers may therefore provide long-term coupling between the growth factor-mediated activation of Ras and subsequent entry of stimulated cells into S phase.

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Role of Ras in signal transduction from the nerve growth factor receptor: relationship to protein kinase C, calcium and cyclic AMP

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A dominant inhibitory *ras* mutant (Ha-*ras* Asn-17) has been used to investigate the role of Ras in nerve growth factor (NGF)-mediated signal transduction in PC12 cells. Expression of Ha-Ras Asn-17 blocks neuronal differentiation of these cells in response to NGF treatment. The Ha-Ras Asn-17 block was bypassed by treatment with NGF plus dibutyryl cAMP or NGF plus the Ca^{2+} ionophore ionomycin, but not by NGF plus 12-*O*-tetradecanoyl phorbol acetate (TPA). Direct stimulation of the cAMP or Ca^{2+} pathways thus appeared to act synergistically with a Ras-independent NGF signaling pathway. This Ras-independent pathway was also distinct from protein kinase C, since its activity was not affected by protein kinase C down-regulation. It thus appears that NGF stimulation generates a Ras-independent intracellular signal that contributes to neuronal differentiation independently of the cAMP, Ca^{2+} or protein kinase C second messenger systems. Since TPA did not bypass the Ha-Ras Asn-17 block to differentiation, protein kinase C also did not appear to be sufficient for Ras-dependent pathways mediating NGF-induced differentiation. Down-regulation experiments further indicated that protein kinase C was not required for NGF induction of early response genes via either Ras-dependent or Ras-independent pathways. Moreover, the formation of inositol phosphates and mobilization of intracellular calcium in response to NGF was not inhibited in PC12 cells expressing the Ha-Ras Asn-17 protein. Therefore, although calcium was able to bypass the Ha-Ras Asn-17 block to PC12 differentiation, Ras activity was not required for activation of phospholipase C in response to NGF. It thus appears that both Ras-dependent and Ras-independent signaling pathways contribute to NGF-induced PC12 cell differentiation independently of the cAMP, calcium and protein kinase C second messenger systems.

Introduction

The mammalian *ras* proto-oncogenes encode small plasma membrane-associated proteins (designated Ras) that bind guanine nucleotides and possess intrinsic GTPase activity, alternating between active GTP-bound and inactive GDP-bound forms (Bourne *et al.*, 1990, 1991; Hall, 1990). Although the function of Ras proteins is not understood, their resemblance to G proteins suggests their involvement in intracellular signal transduction. There is increasing evidence for such a role for Ras not only in mitogenic signal transduction, but also in the process of neuronal differentiation of PC12 pheochromocytoma cells induced by nerve growth factor (NGF).

PC12 cells respond to treatment with NGF by displaying a number of features characteristic of sympathetic neurons, including neurite outgrowth (Greene, 1984). The NGF-induced differentiation of these cells can be mimicked by the action of mutationally activated Ras oncogene proteins (Bar-Sagi & Feramisco, 1985; Noda *et al.*, 1985). The involvement of normal Ras proto-oncogene proteins in PC12 cell differentiation has also been suggested by experiments in which NGF-induced neurite outgrowth was blocked by microinjection of anti-Ras monoclonal antibodies (Hagag *et al.*, 1986). Recently, similar results have been obtained using a dominant inhibitory *ras* mutant (Szeberényi *et al.*, 1990). The protein encoded by this mutant *ras* gene (Ha-Ras Asn-17) preferentially binds GDP versus GTP and strongly inhibits serum- or growth factor-stimulated proliferation of NIH 3T3 cells (Feig & Cooper, 1988; Cai *et al.*, 1990). More recent studies have suggested that the inhibitory activity of Ras Asn-17 correlates with alterations to a Mg^{2+} binding site in the Ras nucleotide-binding domain (Farnsworth & Feig, 1991). Ha-Ras Asn-17 appears to interfere specifically with the function of endogenous proto-oncogene Ras proteins (Stacey *et al.*, 1991), consistent with the behaviour of an analogous yeast *RAS* inhibitory mutant which acts by competing for a guanine nucleotide exchange factor (Powers *et al.*, 1989).

In contrast to its activity in NIH 3T3 cells, Ha-Ras Asn-17 does not affect proliferation of PC12 cells, but instead effectively blocks NGF-induced neuronal differentiation (Szeberényi *et al.*, 1990). Studies of PC12 subclones expressing different levels of Ha-Ras Asn-17 have suggested that Ras is involved in regulation of at least two NGF-stimulated signal transduction pathways (Szeberényi *et al.*, 1990). Cell lines with low-level expression of the mutant Ras protein were resistant to NGF-induced morphological differentiation, but displayed normal induction of early-response genes (*c-fos*, *c-jun* and *zif*268). However, higher levels of Ha-Ras Asn-17 blocked not only the differentiation response, but the NGF induction of early-response genes as well. Thus, it appeared that one signaling pathway required for PC12 differentiation was sensitive to low amounts of Ha-Ras Asn-17, whereas a second pathway, blocked only by high levels of the mutant protein, was sufficient to signal early-

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response gene induction.

Recent studies have identified the protein tyrosine kinase encoded by the *trk* proto-oncogene as a component of the high-affinity NGF receptor (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991). Like other receptor protein tyrosine kinases, the NGF receptor appears to be linked to Ras proteins via association with GTPase-activating protein (GAP) (Ohmichi *et al.*, 1991). NGF treatment of PC12 cells is also known to stimulate phospholipase C (Contreras & Guroff, 1987; Kim *et al.*, 1991), leading to formation of the second messengers diacylglycerol and inositol triphosphate, which activate protein kinase C and mobilize intracellular Ca^{2+} respectively. In addition, NGF treatment results in activation of cAMP-dependent protein kinase (Cremins *et al.*, 1986; Brady *et al.*, 1990). In *Saccharomyces cerevisiae*, RAS proteins activate adenylate cyclase (Toda *et al.*, 1985), but this does not appear to be the case in vertebrates (Beckner *et al.*, 1985). On the other hand, a number of studies have suggested the possibility that Ras proteins may regulate the metabolism of phosphatidylinositides in mammalian cells (Fleischman *et al.*, 1986; Wakelam *et al.*, 1986; Collin *et al.*, 1990; Smith *et al.*, 1990), although these effects have not been shown to result from a direct interaction of Ras with phospholipase C.

The aim of the present study was to further characterize the signaling pathways that mediate the effect of NGF in PC12 cells. We took advantage of the selective inhibition of signal transduction pathways in subclones expressing different amounts of Ha-Ras Asn-17 to investigate the relationships between NGF-stimulated pathways, Ras, and the cAMP, Ca^{2+}, and protein kinase C second messenger systems. The results of these studies establish the existence of a distinct NGF-stimulated pathway that contributes to neuronal differentiation independent of Ras activity. In addition, it appears that both the Ras-dependent and Ras-independent pathways mediating NGF-induced neuronal differentiation are distinct from the cAMP, Ca^{2+} and protein kinase C signal transduction systems.

Results

cAMP and Ca^{2+} induce PC12 cell differentiation in synergy with a Ras-independent signal transduction pathway

To investigate the possible relationships between the cAMP, protein kinase C and Ca^{2+} pathways and Ras, we initially sought to determine whether direct stimulation by second messenger analogs might bypass the Ha-Ras Asn-17 block to NGF-induced differentiation of PC12 cells. Control PC12 cells and a PC12 transfectant subclone expressing high levels of Ha-Ras Asn-17 (M-M17-26 cells) (Szeberenyi *et al.*, 1990) were treated with dbcAMP, TPA or the Ca^{2+} ionophore ionomycin, either alone or in combination with NGF. Treatment with dbcAMP produced a small effect on neurite outgrowth in both PC12 and M-M17-26 cells (Figure 1). A response of similar magnitude was observed upon ionomycin treatment, although processes appeared later than in dbcAMP-treated cells (Figure 1). TPA alone did not induce neurite formation, but increased the effect of dbcAMP to yield levels of differentiation ranging from approximately 20 to 50% in both control and M17 cells (Szeberényi *et al.*, 1990).

NGF alone effectively induced differentiation of control PC12 cells, but not of the Ha-Ras Asn-17-expressing M-M17-26 cells (Figure1). The M-M17-26 cells also failed to differentiate in response to the combination of NGF plus TPA, indicating that direct stimulation of protein kinase C failed to bypass the block to differentiation imposed by expression of the mutant Ras protein (Table 1). In contrast, however, M-M17-26 cells did differentiate in response to either NGF plus dbcAMP or NGF plus ionomycin (Figure 1 and Table 1), although both the fraction of cells differentiated and the length of extended neurites were less than obtained in PC12 cells. The combination of NGF plus dbcAMP displayed similar activity in either medium containing 0.5% serum (Figure 1) or normal growth medium (not shown), whereas ionomycin was only active in low-serum medium. Similar results were obtained with two additional Ha-Ras Asn-17-expressing PC12 subclones, Z-M17-5 and M-M17-2 (Table 1). In both cases, differentiation was effectively induced by NGF plus dbcAMP or by NGF plus ionomycin, but not by NGF plus TPA. It thus appeared that either direct stimulation of cAMP-dependent protein kinase with dbcAMP or increased levels of intracellular Ca^{2+} resulting from treatment with ionomycin bypassed the Ha-Ras Asn-17 block to PC12 cell differentiation.

The action of ionomycin, a Ca^{2+} ionophore, presumably results from increases in the intracellular concentration of Ca^{2+}. To test whether the effect of ionomycin depended on the level of Ca^{2+} in the medium, we performed differentiation experiments with M-M17-26 cells at various Ca^{2+} concentrations (Figure 2). While the synergy between NGF and ionomycin was completely abolished by lowering the Ca^{2+} concentration in the medium, NGF plus dbcAMP (either with or without addition of ionomycin) induced neurite outgrowth even at an extracellular Ca^{2+} concentration of 0.018 mM, which is 100 times lower than that present in Dulbecco's modified Eagle medium (DMEM). The specific dependence of the differentiation-inducing activity of ionomycin on extracellular Ca^{2+} suggests that ionomycin in fact exerts its effect via a Ca^{2+}-mediated mechanism.

Both dbcAMP and ionomycin had to be present in the medium throughout the differentiation process; removal of these agents during the first 3 days of treatment resulted in the disappearance of processes induced by NGF plus dbcAMP or NGF plus ionomycin (data not shown). It thus appeared that continuous stimulation of protein kinase A or Ca^{2+}-dependent protein kinases was required to bypass the block to differentiation imposed by Ha-Ras Asn-17.

While the combination of NGF plus either dbcAMP or ionomycin elicited a strong differentiation response, only a minimal response was evoked by either dbcAMP or ionomycin alone (Figure 1 and Table 1). Thus, the ability of these second messenger analogs to induce differentiation of Ha-Ras Asn-17-expressing PC12 cells appeared to result from synergy with a second intracellular signal generated by the activated NGF receptor. Since this pathway is active in PC12 cells expressing high levels of the Ha-Ras Asn-17 inhibitory mutant protein, it appears to function

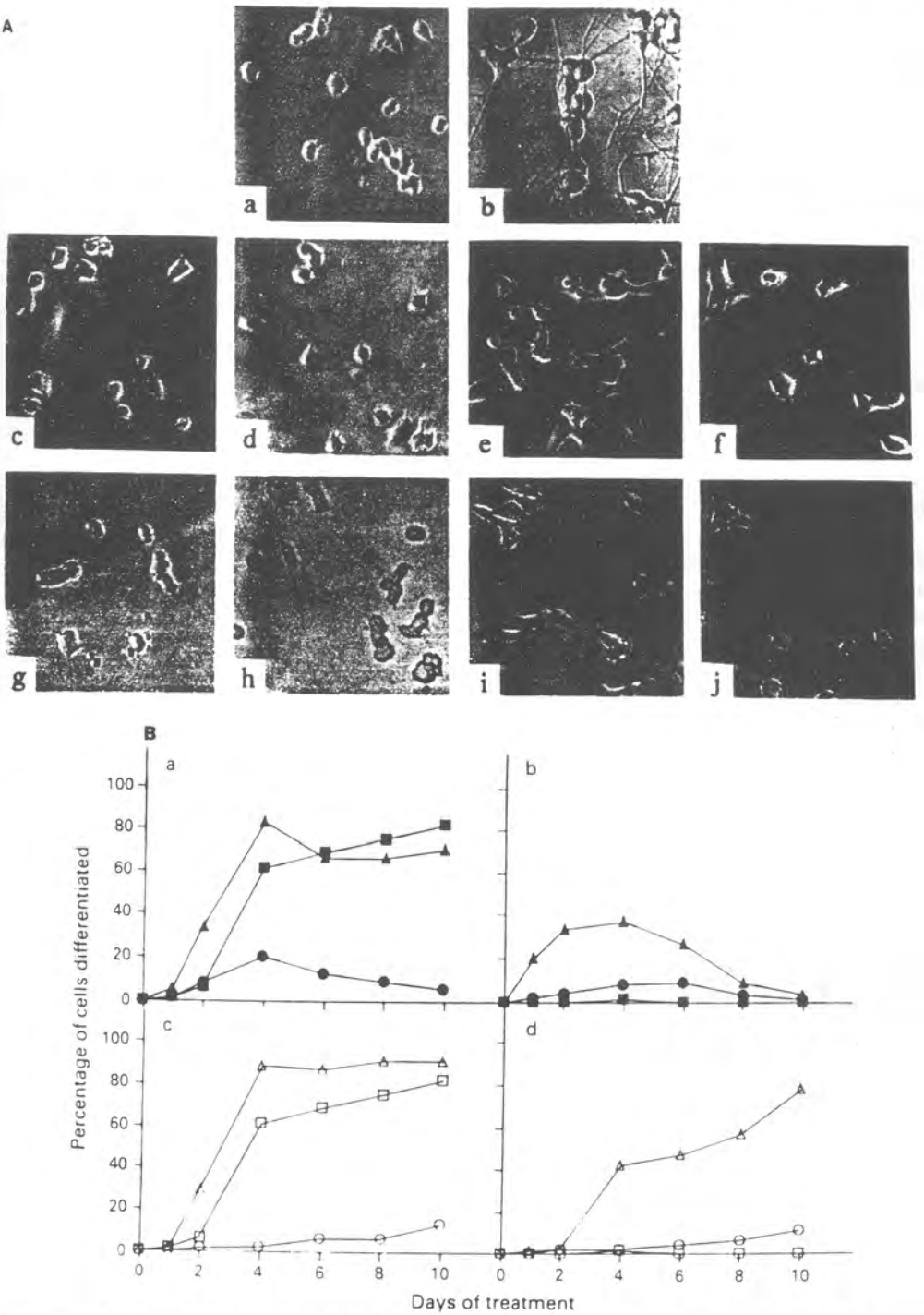

Figure 1 Neuronal differentiation of PC12 and M-M17-26 cells. **(A)** PC12 (a and b) and M-M17-26 (c–j) cells were cultured in DMEM containing 0.5% horse serum without treatment (a, c and g) or in the presence of NGF (b, d and h), dbcAMP (e), ionomycin (i), NGF plus dbcAMP (f) or NGF plus ionomycin (j). Phase contrast micrographs were taken on day 2 (c–f) or day 10 (a, b, g–j) of incubation. Bar, 50 μm. **(B)** Quantitation of a typical differentiation experiment, as described in Materials and methods. PC12 a and c) or M-M17-26 b and d) cells were treated with NGF ■ or □), dbcAMP (●), NGF plus dbcAMP (▲), ionomycin (○) or NGF plus ionomycin (△)

Table 1 Morphological differentiation of PC12 cells expressing Ha-Ras Asn-17

	Percentage of cells differentiated upon treatment with					
Subclone	*NGF*	*dbcAMP*	*Ionomycin*	*NGF + TPA*	*NGF + dbcAMP*	*NGF + ionomycin*
Z-M17-5	3±1 (3)	5±1 (4)	0 (3)	6±1 (4)	25±6 (2)	17±4 (2)
M-M17-2	3±1 (3)	4±1 (3)	0 (1)	3±1 (2)	26±10 (2)	35±13 (2)
M-M17-26	0 (11)	10±2 (7)	7±2 (8)	4±1 (4)	36±3 (6)	48±5 (9)

Cells were treated with the indicated agents for 6 days as described in the legend to Figure 1 and scored for differentiation every other day. The results are expressed as the averages of the highest differentiation scores during the treatment periods in independent experiments ±SEM. The number of experiments for each score is given in parentheses

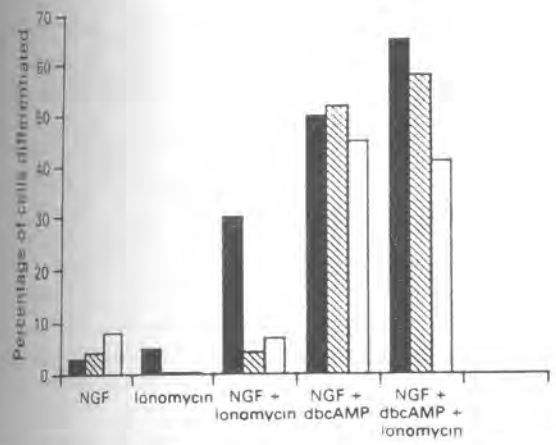

Figure 2 Neuronal differentiation of M-M17-26 cells at various calcium chloride concentrations. Cells were cultured in medium containing 1.8 mM (■), 0.18 mM (▨), or 0.018 mM (□) calcium chloride and treated with the indicated combinations of NGF, dbcAMP and ionomycin for 4 days. Results are presented as the highest differentiation scores during the treatment period

independently of normal Ras activity. Moreover, since this Ras-independent pathway acts synergistically with dbcAMP or Ca^{2+}, it appears to be distinct from pathways involving these second messengers.

The Ras-independent signaling pathway does not require protein kinase C

One candidate for involvement in the NGF-stimulated Ras-independent pathway might be protein kinase C, since this enzyme is activated by NGF treatment of PC12 cells (Cremins *et al.*, 1986; Hama *et al.*, 1986; Heasley & Johnson, 1989). In addition, the combination of dbcAMP plus TPA induces neurite formation in both PC12 cells (Sugimoto *et al.*, 1988) and PC12 cells expressing Ha-Ras Asn-17 (Szeberényi *et al.*, 1990), indicating that activation of protein kinase C can substitute for the Ras-independent NGF signal.

To test the possibility that protein kinase C plays a role in the Ras-independent pathway, the enzyme was down-regulated in M-M17-26 cells by prolonged treatment with TPA (Ballester & Rosen, 1985). In control experiments, such down-regulation of protein kinase C did not inhibit the minimal differentiation induced by dbcAMP alone, but completely abolished the potentiating effect of TPA (Figure 3). This observation indicates that the M-M17-26 cells were effectively depleted of functional protein kinase C by pretreatment with TPA. Such depletion of protein kinase C did not, however, inhibit differentiation induced by NGF plus dbcAMP (Figure 3). Therefore, it appears that protein kinase C is not required for the Ras-independent NGF signaling pathway.

Protein kinase C is not required for induction of early-response genes by NGF

Since TPA plus NGF did not bypass the Ha-Ras Asn-17 block, activation of protein kinase C also does not appear to be sufficient for the activity of Ras-dependent pathways leading to PC12 cell differentiation. However, another possible role for protein kinase C is in the induction of early-response genes. Early-response genes, such as c-*fos*, are induced in PC12 cells by NGF, fibroblast growth factor (FGF) and epidermal growth factor (EGF), as well as by direct activation of protein kinase C by TPA (Greenberg *et al.*, 1985; Bartel *et al.*, 1989). High levels of Ha-Ras Asn-17 (such as expressed in the M-M17-26 subclone) strongly inhibit the induction of c-*fos* by NGF, altough c-*fos* is induced normally in PC12 subclones (such as Z-M17-5 and M-M17-2) that express lower amounts of the mutant Ras protein (Szeberényi *et al.*, 1990). The block to c-*fos* induction in M-M17-26 cells can be bypassed by TPA (Szeberényi *et al.*, 1990), raising the possibility that protein kinase C may be involved in a Ras-dependent pathway leading to early-response gene induction.

To address this question, we studied the induction of c-*fos* by NGF in Z-M17-5 cells, in which a Ras-dependent pathway sufficient to mediate full NGF induction of early-response genes (but not neuronal differentiation) remains functionally intact. To determine whether this pathway is protein kinase C dependent, we studied NGF-stimulated c-*fos* induction in

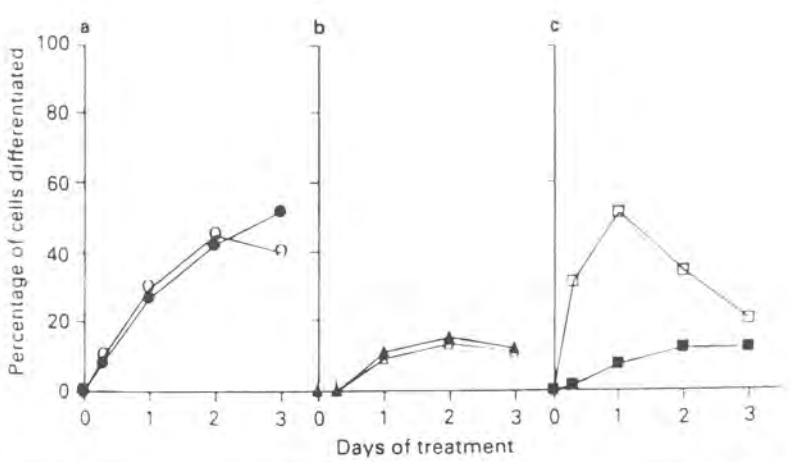

Figure 3 The effect of protein kinase C down-regulation on morphological differentiation of M-M17-26 cells. Protein kinase C was down-regulated by treating the cells with 200 nM TPA for 48 h. Control (open symbols) and protein kinase C down-regulated cells (closed symbols) were treated with NGF plus dbcAMP (○ and ●, **a**), dbcAMP alone (△ and ▲, **b**) or dbcAMP plus TPA (□ and ■; **c**) and scored for morphological differentiation everyday.

Z-M17-5 cells after down-regulating protein kinase C by extended TPA treatment (Figure 4). Down-regulation resulted in the abolition of c-*fos* induction by TPA both in control PC12 and in Z-M17-5 cells. However, NGF induction of c-*fos* was not affected by depleting protein kinase C in either PC12 or Z-M17-5 cells (Figure 4). Similar results were obtained with c-*jun*, *jun*B, *zif*268, *nur*77 and *pip*92 (Lau & Nathans, 1987; Charles *et al.*, 1990) probes (data not shown). It therefore appears that the Ras-dependent NGF signaling pathway can induce a number of early-response genes in the absence of protein kinase C activity.

To further study the possible role of protein kinase C in NGF signaling, we employed transient expression assays using a reporter plasmid containing a TPA-inducible AP-1 sequence element. Expression of pAP1CAT transfected into PC12 cells was induced to a similar extent by either NGF or TPA (Figure 5). The induction by TPA was not affected by co-transfection with a Ha-*ras* Asn-17 expression plasmid, whereas induction by NGF was strongly inhibited. The substantial, but not complete, inhibition of NGF induction of pAP1CAT expression by Ha-Ras Asn-17 is consistent with its effects on NGF induction of endogenous early-response genes (Szeberényi *et al.*, 1990), and suggests that NGF activates AP-1 via both Ras-dependent and Ras-independent pathways. As expected, induction of pAP1CAT by TPA was strongly inhibited by down-regulation of protein kinase C. However, neither the full NGF induction nor the residual NGF induction in the presence of Ha-Ras Asn-17 was affected by protein kinase C down-regulation (Figure 5).

These observations were confirmed using the stably transfected PC12 subclone M-M17-26, which expresses high levels of Ha-Ras Asn-17. NGF induction of pAP1CAT was substantially inhibited in these cells as compared with induction by TPA (Figure 6). However, down-regulation of protein kinase C had no effect on the residual (Ras-independent) induction of pAP1CAT by NGF. Taken together, these experiments indicate that protein kinase C is not required for either the Ras-dependent or the Ras-independent pathways that convey NGF activation of AP-1 elements.

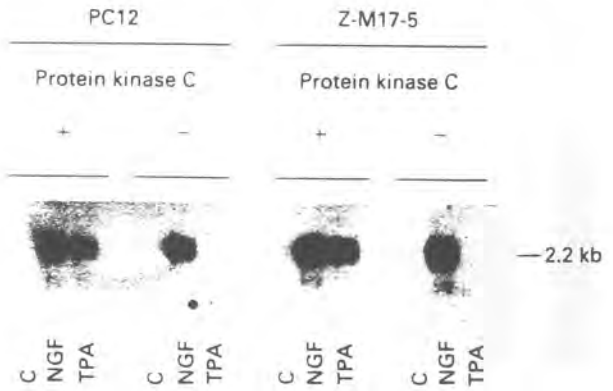

Figure 4 The effect of protein kinase C down-regulation on c-*fos* induction. PC12 and Z-M17-5 cells were plated at a density of 3×10^6 cells per 100-mm dish. Protein kinase C was down-regulated by treatment with 200 nM TPA for 48 h. Both untreated (designated protein kinase C +) and protein kinase C down-regulated (designated protein kinase C −) cultures were then treated with NGF (50 ng ml^{-1}) or TPA (200 nM) for 30 min. Untreated controls are designated C. RNA was isolated, fractionated in an agarose–formaldehyde gel, blotted and hybridized with a ^{32}P-labeled c-*fos* probe. Each sample contained 12 μg of total cytoplasmic RNA

NGF-stimulated inositol phosphate accumulation is not affected by Ha-Ras Asn-17

Our finding that the Ha-Ras Asn-17 block to NGF-induced neuronal differentiation can be, at least partially, bypassed by agonists stimulating cAMP- and Ca^{2+}-regulated pathways is consistent with the hypothesis that these agents might act downstream of the Ras proteins. Alternatively, however, cAMP and Ca^{2+} might stimulate signaling pathways that act in parallel with those normally activated by Ras, thereby

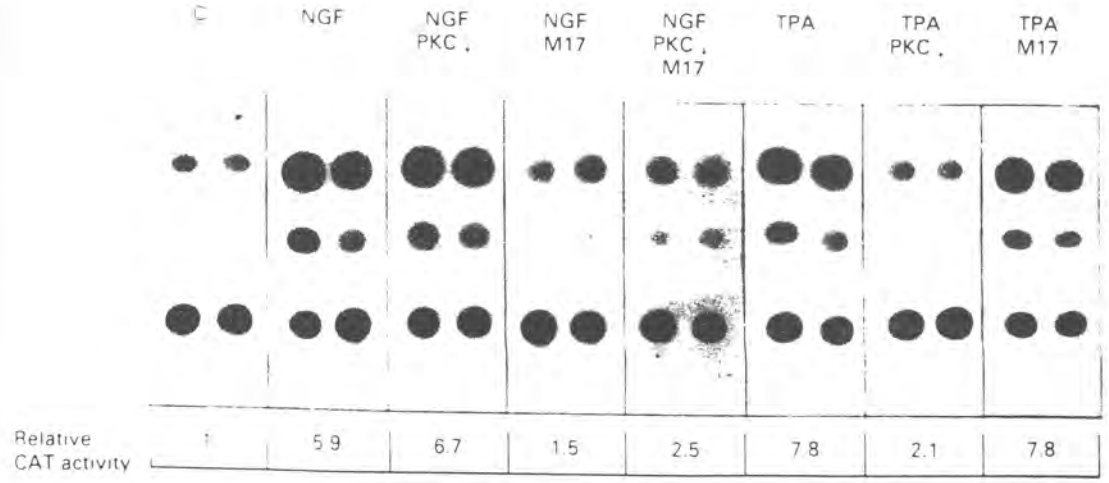

Figure 5 The effect of protein kinase C down-regulation and Ha-Ras Asn-17 expression on AP-1 induction. PC12 cells were transfected with 2 μg of pAP1CAT and 0.5 μg of pMT-Ha-ras Asn-17 (when indicated by M17). Cultures in which protein kinase C was down-regulated are indicated PKC↓. Cells were either maintained as untreated controls (C) or treated with NGF (50 ng ml^{-1}) or TPA (200 nM) for 8 h and assayed for CAT activity

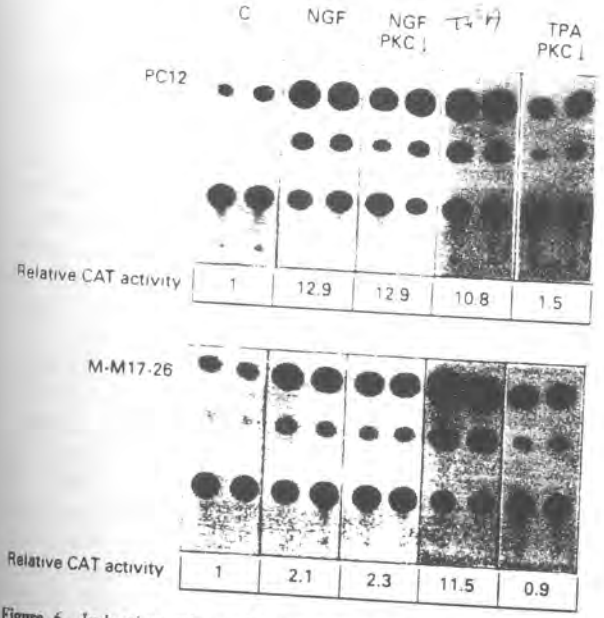

Figure 6 Induction of an AP-1 element in PC12 cells stably expressing Ha-Ras Asn-17. PC12 and M-M17-26 cells were transfected with pAP1TAT and treated as described in the legend to Figure 5

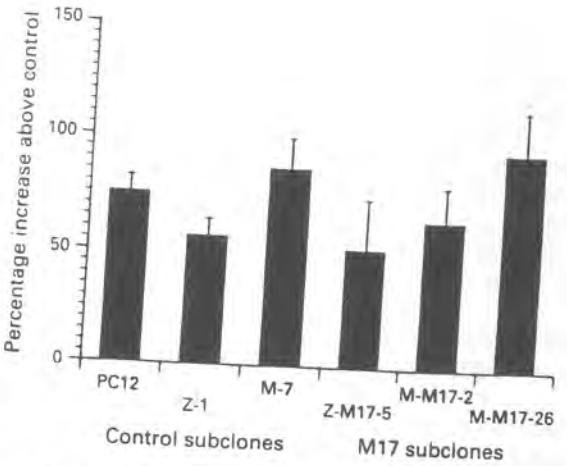

Figure 7 Stimulation of inositol phosphate accumulation by NGF in control and M17 subclones. Control cells (PC12 cells and the Z-1 and M-7 subclones transfected with vector alone) and PC-12 cells expressing Ha-Ras Asn-17 (M17 subclones) were assayed for accumulation of inositol phosphates in response to NGF treatment. Data are presented as the percentage increase of inositol phosphates following a 20-min treatment with NGF in the presence of lithium chloride. Calculation of NGF-stimulated inositol phosphate accumulation was as described in Materials and methods. Each value represents the average of 2–3 independent measurements ± SEM

overcoming a deficiency in Ras activity. Since previous studies have indicated that adenylate cyclase is not regulated by Ras proteins in mammalian cells, we focused on the possible role of Ras proteins in stimulation of phospholipase C, leading to production of inositol triphosphate and mobilization of intracellular calcium.

The accumulation of inositol phosphates in response to NGF treatment was assayed in control and Ha-Ras Asn-17-expressing PC12 subclones (Figure 7). NGF treatment induced a moderate accumulation of inositol phosphates in PC12 cells, consistent with previous studies (Contreras & Guroff, 1987; Kim *et al.*, 1991). Both control and Ha-Ras Asn-17-expressing cells responded to NGF with similar levels of inositol phosphate formation, indicating that Ras function was not required for NGF stimulation of phospholipase C. Furthermore, both PC12 and M-M17-26 cells displayed a moderate, sustained increase in intracellular free calcium following NGF treatment (determined by fluorescence assays of fura-2 AM-loaded cells, data not shown), similar to that previously reported (Pandiella-Alonso *et al.*, 1986; Lazarovici *et al.*, 1989). It thus appears that Ras proteins do not play a role in activation of phospholipase C, and that Ca^{2+} is not acting as a second messenger downstream of Ras.

Discussion

Previous studies using anti-Ras antibodies (Hagag *et al.*, 1986) as well as the dominant inhibitory Ha-*ras* Asn-17 mutant (Szeberenyi *et al.*, 1990) have established the involvement of Ras proto-oncogene proteins in NGF-induced neuronal differentiation of PC12 cells. In the present investigation, we have utilized PC12 cells expressing Ha-Ras Asn-17 to further analyze the involvement of Ras proteins in intracellular signal transduction pathways. These experiments have led to the identification of Ras-independent, as well as Ras-dependent, pathways leading to NGF-induced cell differentiation. Moreover, the intracellular signals that appear critical for induction of differentiation are distinct from cAMP, Ca^{2+} and protein kinase C, implying the utilization of alternative signaling mechanisms in both Ras-dependent and Ras-independent pathways.

The block to NGF-induced neuronal differentiation resulting from Ha-Ras Asn-17 expression can be partially bypassed by direct stimulation of cAMP- or Ca^{2+}-dependent signaling mechanisms, but not by direct stimulation of protein kinase C with TPA. Our finding that the effect of these second messengers is synergistic with NGF establishes the existence of a Ras-independent NGF signaling pathway that may be necessary but not sufficient to induce neuronal differentiation of PC12 cells. Since this Ras-independent pathway acts synergistically with cAMP and Ca^{2+}, it is presumably distinct from these second messengers. In addition, the Ras-independent pathway appears distinct from protein kinase C, since its activity is not affected by protein kinase C down-regulation.

The observation that Ca^{2+} or cAMP can bypass the Ras block suggests the possibility that these second messengers act downstream of Ras in a signal transduction cascade leading to NGF-induced differentiation of PC12 cells. However, the facts that Ras does not use adenylate cyclase as an effector in vertebrate cells (Beckner *et al.*, 1985) and that cAMP-dependent protein kinase is not required for NGF-induced neuronal differentiation (Cremins *et al.*, 1986) make it unlikely that Ras acts to stimulate the cAMP pathway in PC12 cells. On the other hand, several studies have suggested a role for Ca^{2+} in NGF-stimulated neurite outgrowth. The concentration of

inositol phosphates increases following NGF treatment (Contreras & Guroff, 1987; Kim *et al.*, 1991), which also results in a transient increase in intracellular Ca^{2+} levels (Lazarovici *et al.*, 1989; Pandiella-Alonso *et al.*, 1986). In PC12 cells, it has been found that the effects of cAMP and Ca^{2+} converge, both leading to activation of the same transcription factor (CREB) (Dash *et al.*, 1991; Sheng *et al.*, 1991). Because of this convergence, stimulation of the cAMP pathway would be expected to bypass a block to activation of the Ca^{2+} pathway. These considerations are therefore consistent with the hypothesis that Ca^{2+} might act as a second messenger downstream of Ras, as has been suggested by previous studies in which Ras has apparently acted to increase phospholipase C activity (Fleischman *et al.*, 1986; Wakelam *et al.*, 1986; Collin *et al.*, 1990; Smith *et al.*, 1990).

However, direct analysis of the effect of inhibiting Ras function argues against the hypothesis that Ras acts to stimulate phospholipase C in PC12 cells. In particular, the formation of inositol phosphates following NGF treatment as well as the increase in intracellular free Ca^{2+} induced by NGF were unaffected by expression of the Ha-Ras Asn-17 protein. It thus appears that although exogenous Ca^{2+} (or cAMP) can stimulate pathways that bypass the Ras block, neither of these second messengers functions downstream of Ras in NGF-stimulated signal transduction pathways.

Protein kinase C also does not appear to function downstream of Ras in PC12 cells. Direct stimulation of protein kinase C with TPA failed to bypass the Ras block to differentiation, and down-regulation of protein kinase C did not inhibit Ras-dependent induction of early-response genes by NGF. Moreover, protein kinase C down-regulation had no apparent effect on either Ras-dependent or Ras-independent NGF signaling pathways leading to induction of an AP-1 response element. Although it remains possible that protein kinase C is stimulated by Ras activity, it does not appear to play a critical role in the differentiation process.

The identification of the Trk protein tyrosine kinase as a component of the NGF receptor provides a molecular basis for the involvement of Ras proteins in NGF-mediated signal transduction, since Ras proteins are linked to protein tyrosine kinases via GAP (Ohmichi *et al.*, 1991). Other signal transducing molecules that have been found to form complexes with activated protein tyrosine kinase receptors include phospholipase C-γ, phosphatidylinositol-3 kinase, members of the Src family of protein tyrosine kinases, and the Raf protein serine/threonine kinase (Cantley *et al.*, 1991). Phospholipase C-γ is phosphorylated and activated following NGF stimulation (Kim *et al.*, 1991), but (as discussed above) this activation does not appear critical to either Ras-dependent or Ras-independent pathways leading to neuronal differentiation. Recent experiments indicate that the Src protein is required for NGF-induced differentiation, apparently acting as an upstream regulator of Ras activity (Kremer *et al.*, 1991).

The *raf* proto-oncogene product is required for mitogenic signal transduction following stimulation of receptor protein tyrosine kinases (Kolch *et al.*, 1991) and appears to act downstream of Ras in mitogenic signal transduction pathways (Smith *et al.*, 1986; Feig & Cooper, 1988). Similarly, Raf can bypass the Ha-Ras Asn-17 block to PC12 cell differentiation (Jo Troppmair *et al.*, 1992). Moreover, recent experiments indicate that phosphorylation and activation of Raf in both NIH3T3 cells and PC12 cells, as well as MAP kinase in PC12 cells, is blocked by expression of Ha-Ras Asn-17 (Thomas *et al.*, 1992; Wood *et al.*, 1992; Troppmair *et al.*, 1992). Thus, activation of the MAP and Raf protein serine/threonine kinases may be critical events in Ras-dependent signaling.

Phosphatidylinositol-3 kinase may be a candidate for involvement in the Ras-independent signaling pathway. This enzyme is involved in the generation of phosphatidylinositol-3 phosphates, which are thought to function as novel second messengers in mitogenic signal transduction (Cantley *et al.*, 1991). The signal transduction pathway in which these compounds are involved has not yet been well characterized, but the possible involvement of this pathway in Ras-independent NGF-induced signaling merits consideration.

Materials and methods

Cell lines

The PC12-derived cell lines were previously described (Szeberényi *et al.*, 1990). M-M17-26 cells express high levels of Ha-Ras Asn-17, whereas M-M17-2 and Z-M17-5 cells express low levels of the mutant Ras protein. Z-1 and M-7 are control subclones of PC12 cells transfected with the vector alone. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 5% horse serum. Stably transfected cell lines were maintained in medium containing G418 (400 μg ml^{-1}). During the course of experiments, however, G418 was omitted from the medium.

Morphological differentiation assays

Cells were plated in 24-well dishes at a density of 2×10^4 cells per well. On the next day the medium was replaced by DMEM containing 0.5% horse serum and the appropriate differentiation inducers. Agents used for induction of morphological differentiation included NGF (10 ng ml^{-1}; Collaborative Research Inc.), dibutyryl cAMP (dbcAMP) (0.5 mM; Sigma), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (20 nM; Sigma), and ionomycin (0.1–0.25 μM; Calbiochem). In experiments designed to analyse the effect of Ca^{2+} concentration on neurite outgrowth, the cells were stimulated for differentiation in DMEM/Ham's F-12 nutrient mixture (Sigma) supplemented with 2.5 mM glutamine, 0.45 mM leucine, 0.05 mM lysine, 0.12 mM methionine, 0.3 mM magnesium chloride, 0.4 mM magnesium sulfate and various concentrations of calcium chloride. To quantitate the differentiation response, approximately 200 cells were counted in each well, typically every other day of the treatment. Cells with processes longer than their diameter were scored as differentiated.

Northern blot analysis

Isolation of total cytoplasmic RNA, electrophoresis in agarose–formaldehyde gels and hybridization with ^{32}P-labeled probes were performed as described previously (Szeberényi *et al.*, 1990).

Transient expression assays

The reporter plasmid pAP1CAT was constructed by ligating an 18-mer oligonucleotide (5'-GCTTGATGAGTCAGCCG-3')

into the polylinker region of pBLCAT2 (Luckow & Schutz, 1987). The oligonucleotide contains the TPA-inducible AP-1 element of the human collagenase gene (Angel *et al.*, 1987) and drives transcription of the chloramphenicol acetyl transferase (CAT) gene from the herpes simplex virus thymidine kinase promoter. PC12 cells were plated at a density of 7×10^5 cells per 100-mm dish and cultured for 36 h in DMEM containing 10% fetal bovine serum and 5% horse serum. Transfections were performed as described previously (Copeland & Cooper, 1979) except that the cells were incubated with the DNA for 12–15 h. After glycerol shock, cells were incubated for an additional 24 h in DMEM supplemented with 0.5% fetal bovine serum and then treated with NGF (50 ng ml^{-1}) or TPA (200 nM) for 6–8 h. For cultures in which protein kinase C was down-regulated, TPA (200 nM) was included in the medium throughout the entire incubation period. The CAT activity of cell extracts was determined as previously described (Gorman *et al.*, 1982). Reaction mixtures were incubated at 37°C for 4 h and chloramphenicol acetylation was quantitated using a β-scanner.

Phosphoinositide hydrolysis

Accumulation of inositol phosphates in NGF-treated PC12 cells was measured according to Vicentini *et al.*, (1986). Briefly, 2×10^7 cells per 100-mm dish were labeled in inositol-free DMEM containing 0.1% fetal bovine serum and 0.2% horse serum with 20 μCi of [myo-^{3}H]inositol (10–20 Ci mmol^{-1}; New England Nuclear) for 24 h. Cells were collected by scraping with a rubber policeman, resuspended in KR medium (125 mM sodium chloride, 5 mM potassium chloride, 1.2 mM potassium dihydrogen phosphate, 1.2 mM magnesium sulfate, 2 mM calcium chloride, 6 mM glucose, 25 mM Hepes, pH 7.4) containing 10 mM lithium chloride to inhibit inositol phosphate breakdown, and incubated with or without NGF (100 ng ml^{-1}) for 20 min at 37°C in a shaking waterbath. The treatment was terminated by the addition of 1 ml of 20% trichloroacetic acid, 1 mM inositol. Inositol phosphates were separated from other water-soluble inositol derivatives on Dowex AG 1-X8 columns, as described by Horwitz & Perlman (1987). Calculation of control and NGF-stimulated inositol phosphate accumulation was performed as follows. Each [^{3}H]inositol-labeled cell suspension was divided into three samples. Sample A was an untreated control; sample B was treated with 10 mM lithium chloride for 20 min; sample C was treated with 10 mM lithium chloride and 100 ng ml^{-1} NGF for 20 min. Inositol phosphates were isolated from each sample and expressed as the percentage of total radioactivity in the aqueous extracts. The differences between samples B and A and samples C and A give the values for control and NGF-stimulated inositol phosphate accumulations respectively.

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Hydrolysis of Phosphatidylcholine Couples Ras to Activation of Raf Protein Kinase during Mitogenic Signal Transduction

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We have investigated the relationship between hydrolysis of phosphatidylcholine (PC) and activation of the Raf-1 protein kinase in Ras-mediated transduction of mitogenic signals. As previously reported, cotransfection of a PC-specific phospholipase C (PC-PLC) expression plasmid bypassed the block to cell proliferation resulting from expression of the dominant inhibitory mutant Ras N-17. In contrast, PC-PLC failed to bypass the inhibitory effect of dominant negative Raf mutants, suggesting that PC-PLC functions downstream of Ras but upstream of Raf. Consistent with this hypothesis, treatment of quiescent cells with exogenous PC-PLC induced Raf activation, even when normal Ras function was blocked by Ras N-17 expression. Further, activation of Raf in response to mitogenic growth factors was blocked by inhibition of endogenous PC-PLC. Taken together, these results indicate that hydrolysis of PC mediates Raf activation in response to mitogenic growth factors.

The *ras* proto-oncogenes encode guanine nucleotide-binding proteins that play central roles in signal transduction pathways in mammalian cells. A variety of studies have established that Ras proteins are activated in association with protein-tyrosine kinase receptors and are required for growth factor-induced proliferation or differentiation of a number of cell types (4, 10, 11, 25, 32, 36). However, identification of the targets of Ras has remained an outstanding problem in understanding intracellular signal transduction pathways. Recent progress toward this goal has been made by using a dominant inhibitory *ras* mutant (*ras* N-17) (9) to identify intracellular signaling molecules that are activated downstream of Ras in response to growth factor stimulation. These candidate targets include the Raf protein-serine/threonine kinases (9, 33, 40, 45), mitogen-activated protein (MAP) kinase (7, 20, 38, 45), and phosphatidylcholine (PC)-specific phospholipase C (PC-PLC) (3).

Further studies have indicated that MAP kinase lies downstream of Raf in signal transduction pathways, with Raf serving to activate a MAP kinase kinase (MEK) (6, 12, 15). Ras-mediated activation of Raf thus initiates a protein kinase cascade leading to MAP kinase activation. However, although activation of PC hydrolysis in response to growth factor stimulation is also Ras dependent (3), the potential role of PC-derived second messengers in the Raf protein kinase pathway has not been determined.

Hydrolysis of PC is stimulated by a variety of growth factors, providing a second source of diacylglycerol in addition to that derived from hydrolysis of phosphatidylinositides (8, 26). Because mitogen-stimulated turnover of PC persists for several hours after growth factor exposure, it is thought to be particularly important in prolonged signaling leading to long-term responses such as mitogenesis. Hydrolysis of PC is also stimulated by oncogenic Ras proteins (17, 21, 29, 44), and treatment of quiescent fibroblasts with exogenous PC-PLC is itself mitogenic (18). Although hydrolysis of PC can be catalyzed by either PC-PLC or PC-PLD (8), it appears that PC-PLC is the primary source of sustained production of diacylglycerol during the response of fibroblasts to mitogens or Ras transformation (3, 18, 21, 23, 46).

Previous studies have further shown that normal Ras function is required for activation of PC hydrolysis in response to mitogenic stimulation of NIH 3T3 cells (3). Moreover, overexpression of exogenous PC-PLC is able to overcome the block to proliferation of NIH 3T3 cells resulting from inhibition of normal Ras function by the dominant negative mutant Ras N-17 (3). These results indicate that PC-PLC, like Raf, functions downstream of Ras in propagation of mitogenic signals. In the present study, we therefore sought to determine the relationship between PC hydrolysis and activation of the Raf protein kinase. The results indicate that Raf is activated downstream of PC hydrolysis, implying that PC-PLC plays a pivotal role in Ras-mediated mitogenic signaling.

MATERIALS AND METHODS

Cell lines. NIH(M17) cells are a transfected subclone of NIH 3T3 cells in which Ras N-17 is expressed via the glucocorticoid-inducible mouse mammary tumor virus promoter (4). NIH 3T3 and NIH(M17) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum.

Plasmid DNAs. The *ras* expression plasmid pZIP RasN17 contains the Ha-*ras* N-17 gene inserted into the *Bam*HI site of pZIPneoSV(X) so that the mutant *ras* gene is coexpressed with *neo* (9). The dominant negative *raf* mutants, Raf-301 (14) and ΔRaf, were cloned in the pLTRraf vector (35). The ΔRaf mutant was constructed by deletion of codons 283 to 309 from human c-*raf*-1 cDNA. The PC-PLC expression plasmid pOPLCneo was constructed by insertion of the *Bacillus cereus* PC-PLC gene in the mammalian expression

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vector pMAMneo and subsequent deletion of the *neo* gene as previously described (3).

Transfection assays. NIH 3T3 cells were transfected with plasmid DNAs in the presence of 20 μg of carrier calf thymus DNA (Sigma Chemical Co.) as described previously (9). Cells were subcultured into medium containing G418 (400 μg/ml) 3 days after transfection, and G418-resistant colonies were stained and photographed.

PC-PLC and D609. PC-PLC was purified from *B. cereus* as previously described (18). The PC-PLC inhibitor D609 (xanthogenate tricyclodecan-9-yl) was provided by Merz & Co. (Frankfurt). Cells were preincubated with D609 (25 to 35 μg/ml) for 1 h before mitogenic stimulation.

Raf phosphorylation. The activation of Raf in response to mitogenic stimulation was assayed by the electrophoretic mobility shift resulting from Raf-1-activating phosphorylations (40). NIH 3T3 or NIH(M17) cells (10^6 cells per 60-mm-diameter culture dish) were incubated in 0.5% calf serum for 24 h to induce quiescence. Where indicated, expression of Ras N-17 was induced by addition of 5×10^{-7} M dexamethasone. Cells were then labeled for 3 h with $^{32}P_i$ (0.5 mCi/ml) and treated for 20 min with growth factors or PC-PLC. Raf-1 was immunoprecipitated and analyzed by electrophoresis in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels as described previously (40).

Expression of MEK in Sf9 cells. A *Bam*HI-*Not*I fragment containing the entire MEK coding sequence was isolated from a MEK cDNA clone (31) and inserted into pVL1393 transfer vector (Invitrogen). The transfer vector containing the MEK gene (0.5 μg of DNA) was transfected into Sf9 cells along with *Bsu* 361-digested BacPak6 viral DNA (Clontech). Recombinant baculovirus was isolated by plaque purification according to the Clontech BacPak baculovirus expression system product protocol.

For recombinant protein production, 2×10^6 Sf9 cells were infected with MEK virus at a multiplicity of infection of 10 and lysed 48 h postinfection. Sf9 cells were assayed for expression of the recombinant protein by immunoblot analysis. For preparation of MEK, cells were washed twice with ice-cold Tris-buffered saline (137 mM NaCl, 20 mM Tris [pH 7.4]) and lysed in 10 mM Tris-HCl (pH 8.0)–2 mM EGTA–1.5 mM dithiothreitol–5% glycerol–1 mM phenylmethylsulfonyl fluoride–2 μM pepstatin–2 μM leupeptin–10 μg of aprotinin per ml. Insoluble material was removed by centrifugation at 4°C for 5 min at 10,000 × *g*. Octylglucose (1%) was added to the supernatant, and the sample was gently shaken at 4°C. The mixture was centrifuged for 15 min at 100,000 × *g*, and the supernatant was used for MEK preparation as previously described (16).

Raf protein kinase activity. Raf-1 was immunoprecipitated from extracts of 10^6 NIH 3T3 or NIH(M17) cells as previously described (15) except that protein A-Sepharose beads were used. Immunoprecipitates were resuspended in 40 μl of 0.5 mM β-glycerophosphate (pH 7.3)–1.5 mM EGTA–1 mM dithiothreitol–0.03% (wt/vol) Brij 35. Phosphorylation of MEK was then assayed in reaction mixtures (total volume, 76 μl) containing 40 μl of Raf-1 immunoprecipitated beads, 16 μl of Mg-ATP mixture (50 mM $MgCl_2$, 500 μM [γ-^{32}P]ATP; 4,000 to 10,000 cpm/pmol), and 0.1 to 1 μg of MEK. Reaction mixtures were incubated at 30°C for 30 min, the beads were removed by centrifugation, and supernatant fluids were electrophoresed in SDS–10% polyacrylamide gels. Phosphorylation of MEK was quantitated by PhosphorImager scanning.

PC hydrolysis. Stimulation of PC hydrolysis in response to growth factor treatment was assayed by phosphocholine production (3). Cells were prelabeled for 48 h with [^{14}C]choline (2 μCi per culture, 50 to 60 mCi/mmol; New England Nuclear), with the last 24 h of labeling performed in 0.5% calf serum as described above to induce quiescence. Cells were then stimulated with serum or growth factors for 6 h. Choline and phosphocholine were then extracted and analyzed by thin-layer chromatography (3). Phosphocholine was quantitated by using a PhosphorImager.

Phosphorylation of PI-PLCγ. Quiescent cells were stimulated by addition of 10% calf serum for 30 min. Cell lysates were immunoprecipitated with either anti-PI-PLCγ or antiphosphotyrosine monoclonal antibody (UBI, Boston, Mass.). Immunoprecipitates were electrophoresed in SDS–7.5% polyacrylamide gels, and PI-PLCγ was detected by immunoblotting with anti-PI-PLCγ polyclonal antibody (Santa Cruz Biotechnology).

Phosphatidylinositide hydrolysis. Cells were prelabeled for 24 h with [^{3}H]inositol (10 μCi/ml, 10 to 20 Ci/mmol; New England Nuclear) in medium containing 0.5% calf serum. Cells were then washed and incubated in KR medium (125 mM sodium chloride, 5 mM potassium chloride, 1.2 mM potassium phosphate, 1.2 mM magnesium sulfate, 2 mM calcium chloride, 6 mM glucose, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4]) containing 10 mM lithium chloride and D609 (where indicated) for 1 h. Cells were stimulated by addition of 10% calf serum for 20 min, washed, and scraped into 0.5 ml of KR medium plus lithium chloride. Inositol and inositol phosphates were extracted (39), and inositol phosphates were separated from other water-soluble inositol derivates by chromatography on Dowex AG 1-X8 columns (37).

PI 3-kinase assays. Quiescent cells were stimulated by addition of 10% calf serum for 10 min, and cell lysates were immunoprecipitated with antiphosphotyrosine monoclonal antibody (UBI). Immunoprecipitates were then assayed for phosphatidylinositol (PI) 3-kinase activity by incubation in the presence of PI and [^{32}P]ATP (1). Reaction mixtures were analyzed by thin-layer chromatography, and formation of PI 3-phosphate was quantitated by using a PhosphorImager.

RESULTS

PC-PLC bypasses the inhibition of cell proliferation resulting from expression of dominant negative *ras* but not *raf* mutants. Previous experiments demonstrated that overexpression of PC-PLC could overcome the inhibition of cell proliferation resulting from expression of the dominant negative Ras N-17 mutant in cotransfection assays (3). We therefore initially investigated the relationship between PC-PLC and Raf by testing whether overexpression of PC-PLC could similarly bypass the inhibitory activity of dominant negative Raf mutants. Results of a representative experiment are illustrated in Fig. 1, and data from three independent experiments are summarized in Fig. 2.

As previously reported (9), transfection of the dominant inhibitory *ras* N-17 mutant with a neomycin resistance gene inhibited the proliferation of transfected NIH 3T3 cells, as indicated by the reduction in G418-resistant transformants obtained following transfection with the pZIP RasN17 plasmid compared with the pZIPneoSV(X) vector alone. A comparable reduction in G418-resistant colonies was observed following cotransfection of pZIPneoSV(X) with plasmids directing the expression of dominant negative Raf proteins. Similar results were obtained with two different inhibitory *raf* mutants: the previously characterized kinase-deficient mutant Raf-301 (14) and an additional inhibitory

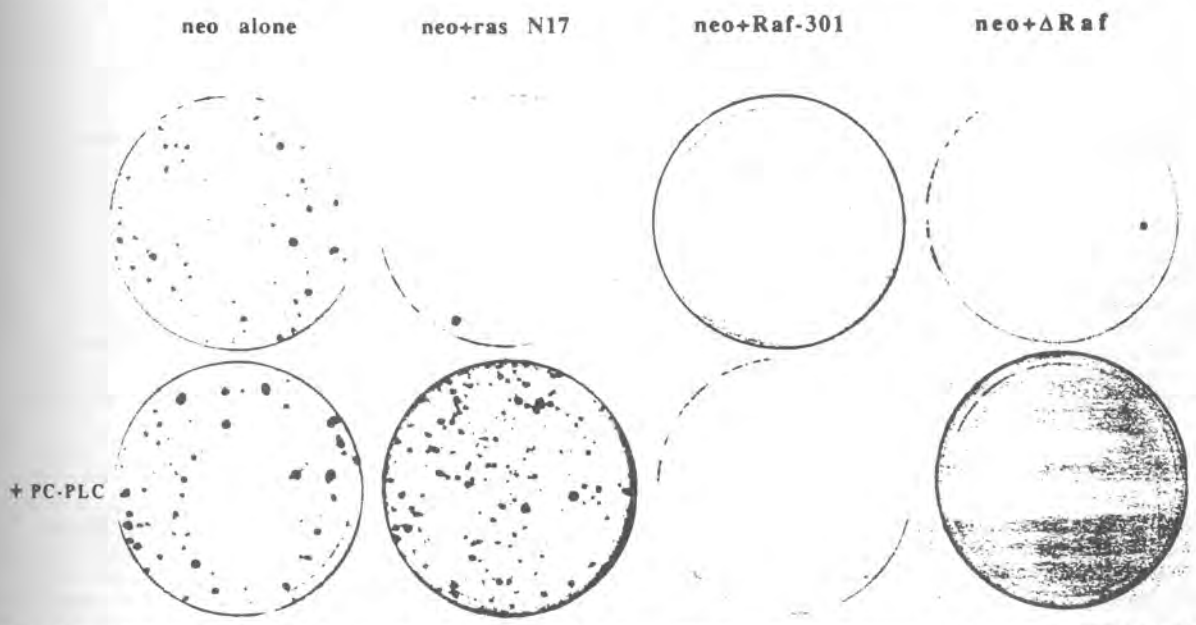

FIG. 1. Cotransfection with PC-PLC does not bypass inhibition of cell proliferation by dominant negative Raf mutants. NIH 3T3 cells were transfected with the indicated plasmid DNAs and subcultured into medium containing G418. Cultures: neo alone, transfected with 0.1 μg of pZIPneoSV(X); neo+ras N-17, transfected with 0.1 μg of pZIP RasN17; neo+Raf-301 and neo+ΔRaf, cotransfected with 3 μg of the Raf expression plasmids plus 0.1 μg of pZIPneoSV(X); + PC-PLC, transfected with the plasmids described above plus 5 μg of the PC-PLC expression vector pOPLCneo . G418-resistant colonies were stained and photographed 14 days after transfection.

mutant (ΔRaf) resulting from deletion of Raf codons 283 to 309, which encompass multiple sites of Raf phosphorylation (22).

Cotransfection with a PC-PLC expression plasmid reversed the inhibitory effect of Ras N-17, indicating that overexpression of PC-PLC could overcome the block to cell proliferation resulting from inhibition of normal Ras function (3). In contrast, cotransfection with PC-PLC failed to reverse the growth-inhibitory effects of the dominant negative Raf mutants, indicating that PC-PLC is unable to bypass the inhibition of cell proliferation resulting from interference with normal Raf activity. It thus appears that PC-PLC acts downstream of Ras, but upstream of Raf, in mitogenic signaling.

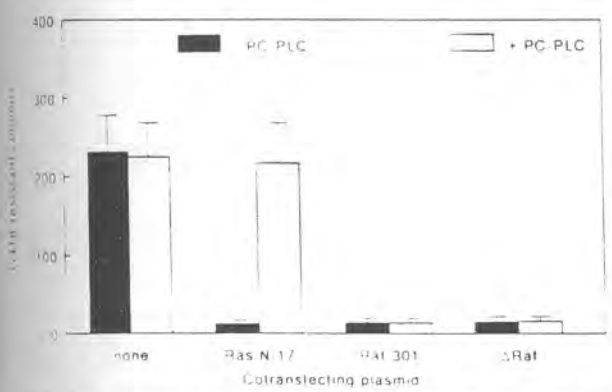

FIG. 2. Cotransfection with PC-PLC and dominant negative Raf mutants. NIH 3T3 cells were transfected and cultured as described in the legend to Fig. 1. Data from three independent experiments, each with triplicate plates, are presented as the number of G418-resistant colonies per plate ± standard deviation.

Activation of Raf by treatment with exogenous PC-PLC. The possibility that PC hydrolysis serves to couple Ras to Raf activation was further investigated by analysis of Raf phosphorylation following treatment of quiescent NIH 3T3 cells with PC-PLC. Under conditions previously found to be mitogenic (3, 18), treatment with exogenous PC-PLC purified from *B. cereus* induced the electrophoretic mobility shift characteristic of Raf activation (Fig. 3). The effects of PC-PLC treatment on Raf were similar to the effects of treatment with epidermal growth factor (EGF) or the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig. 3). These results indicated that PC hydrolysis, like mitogenic growth factors or phorbol esters, induces hyperphosphorylation characteristic of the activation of Raf protein kinase.

Activation of Raf in response to serum, EGF, and TPA is dependent upon normal Ras activity and is blocked by Ras N-17 expression (40, 45). However, if PC-PLC acts downstream of Ras to activate Raf, one would predict that its activity would not be inhibited by expression of the dominant negative Ras mutant. We therefore investigated Raf activation in the NIH(M17) cell line compared with normal NIH 3T3 cells (Fig. 3). As previously reported (40), induction of Ras N-17 by dexamethasone treatment blocked Raf hyperphosphorylation in response to EGF and TPA. In contrast, induction of Ras N-17 did not affect Raf hyperphosphorylation following treatment with PC-PLC.

To verify that these effects on Raf activation assessed by hyperphosphorylation coincided with Raf protein kinase activity, we assayed Raf-1 phosphorylation of MEK substrate (Fig. 4). The protein kinase activity of Raf-1 was increased approximately 10-fold in response to stimulation of quiescent cells with either EGF or PC-PLC. Consistent with the assays of Raf activation by hyperphosphorylation, induction of Ras N-17 by dexamethasone blocked stimulation of Raf-1 protein kinase activity by EGF but not by

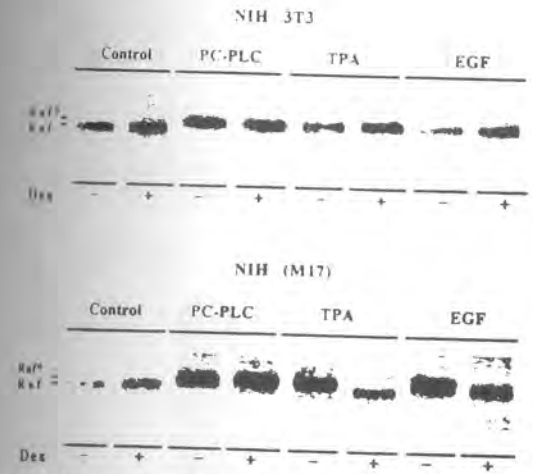

FIG. 3. Induction of Raf-1 phosphorylation by exogenous PC-PLC. NIH 3T3 or NIH(M17) cells were made quiescent by incubation in 0.5% calf serum for 24 h. Where indicated, expression of Ras N-17 in the NIH(M17) cells was induced by addition of 5×10^{-7} M dexamethasone (Dex). Cells were then labeled for 3 h with $^{32}P_i$ (0.5 mCi/ml) and treated for 20 min with EGF (10 ng/ml), TPA (100 nM), or PC-PLC purified from *B. cereus* (0.5 U/ml). Raf-1 was immunoprecipitated and analyzed by electrophoresis in SDS–7.5% polyacrylamide gels. The band designated Raf* represents the electrophoretic mobility shift characteristic of Raf-1-activating phosphorylations.

PC-PLC. It therefore appears that a PC-derived second messenger can activate Raf in the absence of Ras activity, consistent with PC hydrolysis lying between Ras and Raf in the mitogenic signaling pathway.

Inhibition of cellular PC-PLC inhibits Raf activation. In a third approach to analyzing the relationship between PC-PLC and Raf, we investigated the effects of inhibiting

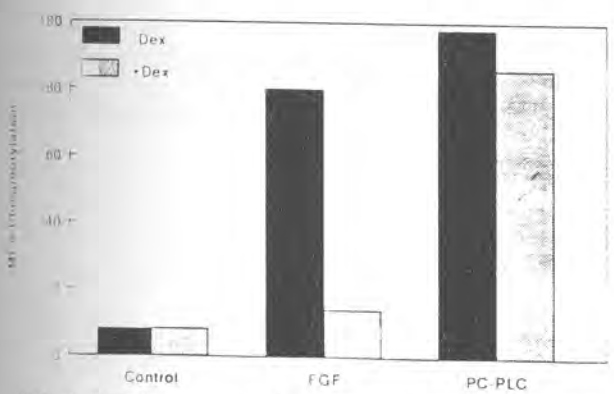

FIG. 4. Stimulation of Raf-1 protein kinase by PC-PLC. NIH(M17) cells were incubated in 0.5% calf serum for 24 h. Expression of Ras N-17 was induced by dexamethasone (Dex), and cells were either left unstimulated (control) or treated for 20 min with EGF or PC-PLC as for Fig. 3. Raf-1 was immunoprecipitated and assayed for protein kinase activity by phosphorylation of MEK purified from baculovirus-infected Sf9 cells. Reaction mixtures were electrophoresed in SDS–10% polyacrylamide gels, and MEK phosphorylation was quantitated by PhosphorImager scanning (arbitrary units are shown). Control reaction mixtures either lacking MEK or containing immunoprecipitates with preimmune rather than anti-Raf antiserum had less than 10% of the stimulated levels of MEK phosphorylation.

	Control	Serum	EGF	Control	Serum	EGF
D609	−	−	−	+	+	+
Fold Stimulation	1	9.5	10.7	1	1.5	0.8

FIG. 5. Inhibition of PC hydrolysis by D609. Quiescent NIH 3T3 cells were prelabeled with [^{14}C]choline and preincubated for 1 h with D609 (25 μg/ml). Duplicate cultures were then stimulated by addition of 10% calf serum or EGF for 6 h. Choline and phosphocholine were extracted and analyzed by thin-layer chromatography. Phosphocholine was quantitated by using a PhosphorImager, and the average fold stimulation of phosphocholine production by mitogens is shown at the bottom.

endogenous PC-PLC on Raf activation in response to mitogens. For these experiments, we used the compound D609, which has been reported to specifically inhibit PC-PLC without inhibiting other phospholipases, including PC-PLD, PLA_2, or PI-PLC (30). Treatment of quiescent NIH 3T3 cells with D609 inhibited stimulation of PC hydrolysis in response to serum or EGF (Fig. 5), indicating that this compound is an effective inhibitor of growth factor-stimulated PC-PLC in NIH 3T3 cells.

As controls for the specificity of the inhibitor, we investigated the effects of D609 on other growth factor-stimulated activities which have previously been shown to be independent of Ras function, namely, the tyrosine phosphorylation and activation of PI-PLCγ and PI 3-kinase (7, 37, 38, 40, 45). Tyrosine phosphorylation of PI-PLCγ in serum-stimulated cells was analyzed by immunoprecipitation of cell lysates with antiphosphotyrosine antibody. PI-PLCγ was detected by immunoblotting of such immunoprecipitates from serum-stimulated, but not quiescent, NIH 3T3 cells (Fig. 6). The tyrosine phosphorylation of PI-PLCγ following serum stimulation was unaffected by D609 (Fig. 6), indicating that D609 did not inhibit receptor tyrosine kinase activity.

To determine whether D609 inhibited the intracellular activity of PI-PLCγ, we also investigated its effect on phosphatidylinositide hydrolysis following mitogenic stimulation. The formation of inositol phosphates was increased approximately fivefold in response to serum stimulation of quiescent NIH 3T3 cells (Fig. 6). This was unaffected by D609 addition, indicating that D609 did not detectably inhibit the intracellular activity of PI-PLCγ.

Consistent with its lack of effect on the phosphorylation and activation of PI-PLCγ, D609 also failed to inhibit tyrosine phosphorylation of PI 3-kinase, as assessed by PI 3-kinase assays of antiphosphotyrosine immunoprecipitates (Fig. 7). Thus, consistent with findings of previous studies (30), D609 appears to inhibit PC-PLC without interfering with the activation of other signal-transducing proteins via tyrosine phosphorylation or with the hydrolysis of phosphatidylinositides catalyzed by PI-PLC.

Since D609 appeared to be a specific inhibitor of intracellular PC hydrolysis, it was then used to investigate the effect of inhibiting PC-PLC on Raf activation in response to growth factors. As illustrated in Fig. 8, treatment of quiescent NIH 3T3 cells with D609 effectively inhibited Raf phosphorylation in response to serum, EGF, and TPA. Likewise, D609 blocked the stimulation of Raf protein kinase activity by either serum or EGF (Fig. 9). It thus appears that PC-PLC is

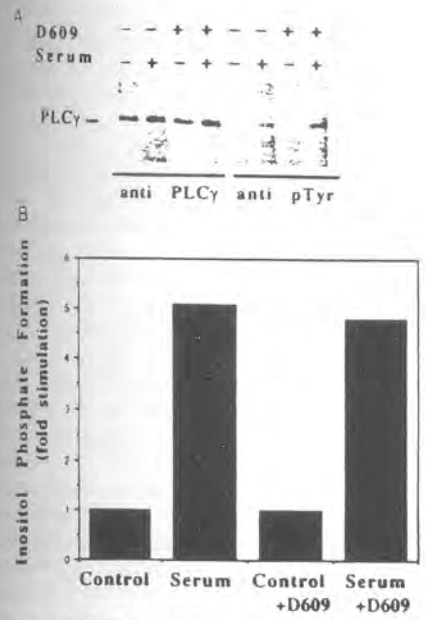

FIG. 6. Lack of effect of D609 on tyrosine phosphorylation and activity of PI-PLCγ. (A) Quiescent NIH 3T3 cells were preincubated with D609 (35 μg/ml) for 1 h and then stimulated with 10% calf serum for 30 min. Cell lysates were immunoprecipitated with either anti-PI-PLCγ or anti-phosphotyrosine (anti pTyr) monoclonal antibody. Immunoprecipitates were electrophoresed in SDS-7.5% polyacrylamide gels, and PI-PLCγ was visualized by immunoblotting with anti-PI-PLCγ polyclonal antibody. (B) Cells were prelabeled for 24 h with [^{3}H]inositol (10 μCi/ml) in medium containing 0.5% calf serum. Cells were then washed and incubated in KR medium containing 10 mM lithium chloride and D609 (35 μg/ml) for 1 h. Cells were stimulated by addition of 10% calf serum for 20 min, washed, and scraped into 0.5 ml of KR medium plus lithium chloride. Inositol and inositol phosphates were extracted, and inositol phosphates were isolated by chromatography on Dowex AG 1-X8 columns. Data from a representative experiment are presented as the fold increase in inositol phosphate production in response to mitogen stimulation.

required for Raf activation in response to mitogenic stimuli, indicating that PC hydrolysis serves to couple Ras to activation of the Raf protein-serine/threonine kinase.

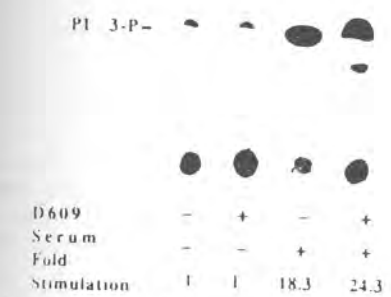

FIG. 7. Lack of effect of D609 on PI 3-kinase. Quiescent NIH 3T3 cells were preincubated with D609 (35 μg/ml) and then stimulated with 10% calf serum for 10 min. Cell lysates were immunoprecipitated with antiphosphotyrosine monoclonal antibody. Immunoprecipitates were then assayed for PI 3-kinase activity by incubation in the presence of PI and [γ-^{32}P]ATP. The formation of PI 3-phosphate was quantitated by using a PhosphorImager, and the fold stimulation in response to mitogens is shown at the bottom.

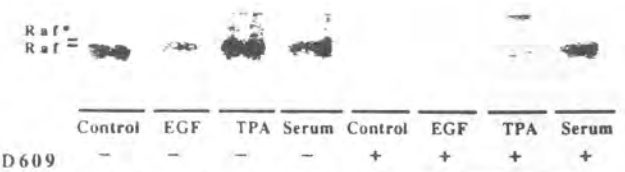

FIG. 8. Inhibition of Raf-1 phosphorylation by D609. Activating phosphorylation of Raf-1 in quiescent NIH 3T3 cells treated with EGF, TPA, or 10% calf serum was assayed as for Fig. 3. Where indicated, cells were preincubated for 1 h with D609 (35 μg/ml).

DISCUSSION

Previous studies have established that growth factor stimulation of PC hydrolysis is mediated by Ras and that PC-PLC can act downstream of Ras to induce cell proliferation (3, 18, 21). In the present study, we have investigated the role of PC hydrolysis with respect to activation of the Raf protein-serine/threonine kinase. Three lines of experimentation indicate that PC-PLC acts upstream of Raf, with production of a PC-derived second messenger, presumably diacylglycerol, leading to Raf activation. First, overexpression of PC-PLC in cotransfection assays was able to overcome the block to cell proliferation resulting from expression of dominant negative Ras but not Raf. Thus, in these functional mitogenesis assays, PC-PLC acts downstream of Ras but upstream of Raf. Second, treatment of quiescent cells with exogenous PC-PLC led to Raf activation. Importantly, Raf activation by PC-PLC was not inhibited by expression of the dominant negative Ras N-17 mutant, indicating that PC hydrolysis was able to activate Raf in the absence of normal Ras activity. Third, we used the PC-PLC inhibitor D609 to inhibit PC hydrolysis in mitogen-stimulated cells. D609 specifically inhibited PC hydrolysis without affecting tyrosine phosphorylation of PI-PLCγ or PI 3-kinase, nor did it inhibit hydrolysis of phosphatidylinositides by PI-PLCγ. However, D609 effectively inhibited the activation of Raf in response to mitogen stimulation. Taken together, these results indicate that PC hydrolysis acts upstream of Raf and serves to couple Ras to Raf activation during mitogenic signaling.

The nature of the coupling between PC-PLC and Raf

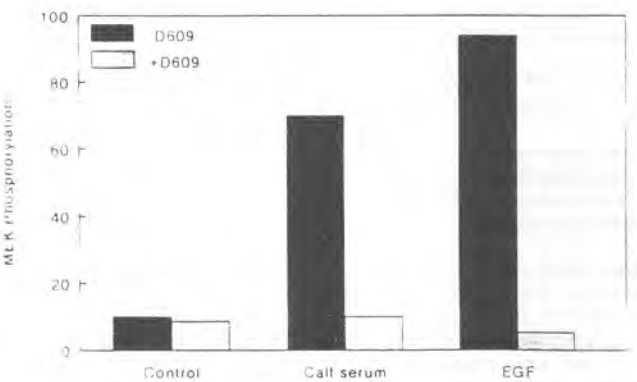

FIG. 9. Inhibition of mitogen stimulation of Raf-1 kinase activity by D609. Phosphorylation of MEK by Raf-1 immunoprecipitates was assayed as for Fig. 4. Quiescent NIH 3T3 cells were stimulated with 10% calf serum or EGF and, where indicated, preincubated for 1 h with D609 (35 μg/ml). MEK phosphorylation is expressed as arbitrary PhosphorImager units.

remains to be established. It is possible that PC-derived diacylglycerol serves directly as a Raf activator, possibly by binding to the Raf amino-terminal regulatory domain, which contains a zinc finger motif similar to the ligand-binding domain of protein kinase C and may function to bind a Raf-activating factor (2). It is noteworthy in this regard that the species of diacylglycerol derived from hydrolysis of PC differ from those derived from phosphatidylinositides and may have distinct second-messenger activities (19, 27, 28). Although none of the Raf enzymes bind phorbol-12,13-dibutyrate, which is a standard ligand for classical forms of protein kinase C, the pattern of binding and kinase activation by PC-derived diacylglycerol remains to be established.

Alternatively, PC-derived diacylglycerol might activate other protein kinases which then activate Raf via phosphorylation. Consistent with this possibility, recent studies have demonstrated activation of Raf by several protein kinase C isotypes (13, 34). Importantly, however, downregulation of protein kinase C by prolonged treatment with phorbol esters inhibits neither the mitogenic activity of PC-PLC (18) nor the activation of Raf by Ras (5). Therefore, if protein kinase C mediates Raf activation, a nonclassical isoform resistant to phorbol ester downregulation would have to be involved (26). Alternatively, PC-derived diacylglycerol might activate yet another class of Raf kinase kinases.

It should also be noted that in contrast to its activity in NIH 3T3 cells, PC-PLC may not be involved in Raf activation during Ras-mediated differentiation of PC12 cells. In these cells, we have not observed reproducible stimulation of PC turnover in response to nerve growth factor, nor is Raf activation inhibited by D609 (data not shown). This might be due to technical factors, such as differences in drug permeability or phospholipid pools between the two cell types. Moreover, PC12 cells are a tumorigenic cell line in which some signaling pathways may be constitutively activated. It is also possible, however, that a different pathway serves to couple Ras to Raf during neuronal differentiation of this cell line.

Several groups have recently reported direct protein-protein interactions between Ras and Raf, which are presumably involved in Raf activation (24, 41–43, 47). However, the potential effect of such interactions on Raf kinase activity has not been determined. It is possible that interactions between Ras and Raf mediate the association of Raf with a signaling complex where it is then activated as a result of PC hydrolysis. Alternatively, direct Ras-Raf protein interactions and PC hydrolysis might represent independent modes of activating the Raf protein kinase.

Previous studies have indicated that Ras is required for the activation of PC-PLC, Raf, and MAP kinase in response to growth factor stimulation (3, 7, 35, 40, 45) and that MAP kinase is activated downstream of Raf (6, 12, 15). The present results allow the placement of PC hydrolysis upstream of the Raf protein kinase cascade in a linear pathway of mitogenic signal transduction. The role of PC hydrolysis in coupling Ras to Raf further indicates that PC-PLC occupies a central position in Ras-mediated mitogenic signaling.

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Rapid Communication

ras-Independent Induction of Rat Brain Type II Sodium Channel Expression in Nerve Growth Factor-Treated PC12 Cells

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Abstract: Nerve growth factor (NGF) plays an important role in the development of the nervous system, and there is considerable interest in understanding the molecular mechanisms underlying its effects on neuronal differentiation. To determine if the activity of proteins of the *ras* gene family is necessary for the NGF-mediated induction of sodium channel expression in pheochromocytoma (PC12) cells, sodium channel expression was analyzed in PC12 sublines stably overexpressing the dominant inhibitory mutant c-Ha-*ras*(Asn-17). Northern blot analysis, RNase protection assays, and whole-cell patch clamp recordings indicate that the NGF-mediated increase in type II sodium channel mRNA and sodium current density can occur independent of *ras* activity and by doing so provide strong evidence for the importance of *ras*-independent mechanisms in NGF-mediated neuronal differentiation. **Key Words:** Neuronal differentiation—Nerve growth factor—Sodium channels—*ras*—Gene expression—Patch clamping.
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Nerve growth factor (NGF) plays a crucial role in the development of the nervous system (for review, see Wagner and Kostyk, 1991). As a result, there is considerable interest in identifying molecular mechanisms underlying the actions of NGF, especially those associated with neuronal differentiation, that distinguish the biological actions of NGF from growth factors such as epidermal growth factor. Differences in the actions of these growth factors are evident even within the same cell type, as exemplified in rat pheochromocytoma (PC12) cells, where NGF causes cessation of cell division and induces a sympathetic neuron-like phenotype, whereas epidermal growth factor causes proliferation and fails to elicit many of the neuronal-specific changes in phenotype (Huff et al., 1981; for review, see Chao, 1992).

An important aspect of the NGF-induced differentiation of PC12 cells is the increased expression of voltage-dependent Na^+ channels (Dichter et al., 1977; Mandel et al., 1988), which play a central role in the generation of action potentials (Hodgkin and Huxley, 1952). The principal component of Na^+ channels is an ~260-kDa α subunit (for review, see Stephan and Agnew, 1991). At least four α subunit genes (type I, II, IIa, and III) are expressed in the nervous system, each exhibiting a distinct pattern of expression (Beckh et al., 1989; for review, see Mandel, 1992). The molecular mechanisms governing Na^+ channel expression are largely unknown, despite the fact that an analysis of neuronal Na^+ channel induction by NGF may be more informative than an analysis of other more general responses when trying to identify the crucial steps distinguishing the response to NGF from the responses to other growth factors (for discussion, see Chao, 1992).

Among the proteins implicated in the signaling pathways activated by NGF are the 21-kDa proteins of the *ras* gene family (p21*ras*) (for review, see Haleguoa et al., 1991), which are necessary for many NGF-induced responses (Szeberenyi et al., 1990; Kremer et al., 1991; Wood et al., 1992; Borasio et al., 1993). However, not all responses to NGF appear to require *ras* activity (Szeberenyi et al., 1992; Borasio et al., 1993; for discussion, see Chao, 1992), and the exact role of *ras* in neuronal differentiation has not been established. Therefore, to determine if p21*ras* activity is necessary for NGF-induced Na^+ channel expression in PC12 cells, we have analyzed Na^+ channel expression in PC12 sublines stably overexpressing the dominant inhibitory *ras* mutant c-Ha-*ras*(Asn-17) (Szeberenyi et al., 1990).

MATERIALS AND METHODS

Cell culture

Cells were plated and maintained in 35- (for electrophysiology) or 100-mm diameter (for RNA) culture dishes (Falcon Labware, Becton Dickinson, Lincoln Park, NJ, U.S.A.) for 7 days, as previously described (Ginty et al., 1992), with some receiving 100 ng/ml of 7S NGF (Upstate Biotechnology, Plattsburg, NY, U.S.A.) every other day when the medium was changed.

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Abbreviations used: MAPK, mitogen activated protein kinase; NGF, nerve growth factor; RSK, ribosomal S6 kinase.

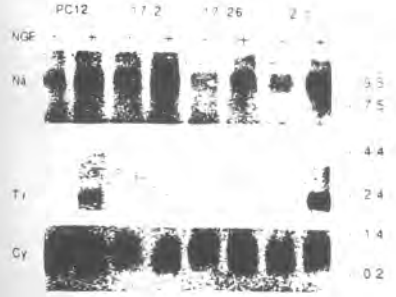

FIG. 1. Representative northern blots of Na⁺ channel (Na), transin (Tr), and cyclophilin (Cy) mRNAs in 40-μg samples of total cellular RNA from untreated (−) and NGF-treated (+) PC12, MM17-2, MM17-26, and Z-1 cells. The positions and molecular sizes of a commercially available RNA ladder (GIBCO BRL) are shown on the right. The Na⁺ channel and transin signals are shown after a 24-h exposure to film. The cyclophilin mRNA signals are shown after a 30-min exposure to film.

RNA isolation and analysis

Isolation of total cellular RNA and northern blot analysis of Na⁺ channel, cyclophilin, and transin mRNAs were done as previously described (Szeberenyi et al., 1990; Ginty et al., 1992), except that a commercially available random-primer DNA labeling kit (GIBCO BRL, Grand Island, NY, U.S.A.) and [^{32}P]CTP (Amersham) were used to generate the probe for transin mRNA. RNase protection analysis was performed as described by Hod (1992). Templates for cRNA probes specific for rat brain type I, II, IIa, and III Na⁺ channel mRNA were constructed as follows: Complementary oligonucleotides (Operon Technologies, Alameda, CA, U.S.A.) corresponding to gene-specific sequences in the 3′ untranslated regions of the type I, II, IIa, or III rat brain Na⁺ channel gene (Auld et al., 1988; Beckh et al., 1989) were annealed and ligated into pGEM-4Z (Promega, Madison, WI, U.S.A.). The template for the type I-specific probe corresponded to nucleotides 7158–7217 of the published cDNA sequence, the type II template to nucleotides 6624–6700, the type IIa template to nucleotides 6415–6495, and the type III template to nucleotides 6072–6151 (Noda et al., 1986; Auld et al., 1988; Kayano et al., 1988). A second type II-specific probe was generated from a genomic DNA fragment containing 5′ untranslated and coding sequences, as previously described (Mandel et al., 1988). The cyclophilin-specific probe was generated from a template corresponding to nucleotides 306–342 of the published sequence (Danielson et al., 1988). cRNA probes were generated using a commercially available kit (Promega) and [^{32}P]UTP (Amersham) and were gel-purified before 20 μg of total cellular RNA was hybridized with 2.5×10^5 dpm of the Na⁺ channel and cyclophilin probes at 46°C for 12–16 h. After digestion with 40 μg/ml of RNase A and 2 μg/ml of RNase T1 (United States Biochemical, Cleveland, OH, U.S.A.), protected fragments were separated on a 6% acrylamide gel and exposed to Kodak XAR film for 24 h at −80°C. The NIH IMAGE program was used for densitometric analysis of the autoradiographic signals representing Na⁺ channel and cyclophilin mRNA fragments.

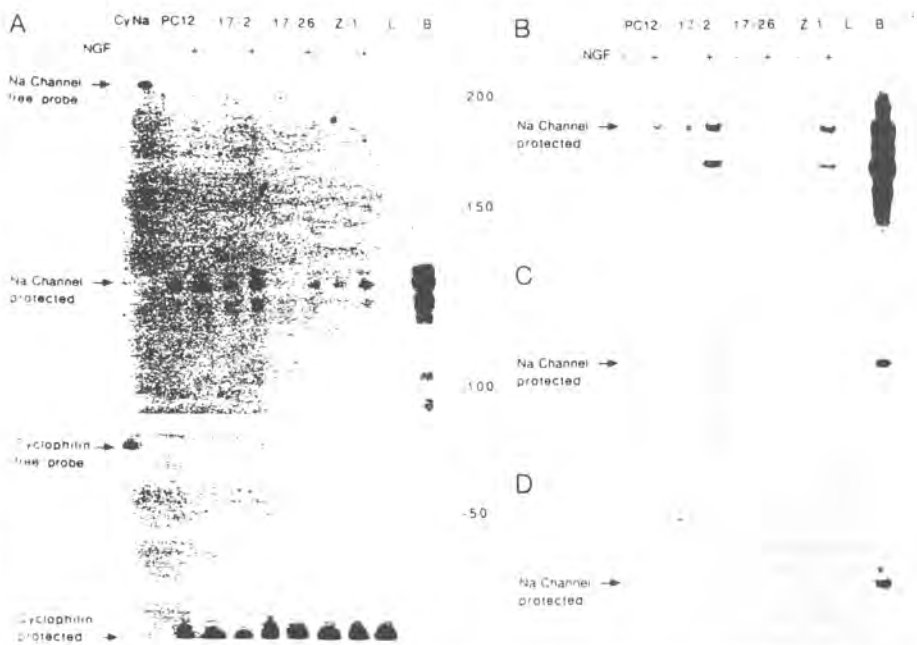

FIG. 2. RNase protection analysis of type-specific Na⁺ channel mRNA. Total cellular RNA (20 μg) from untreated (−) and NGF-treated (+) PC12, MM17-2, MM17-26, and Z-1 cells, from rat liver (L), or from adult rat brain (B) was incubated with both a probe for a specific Na⁺ channel mRNA and a probe specific for cyclophilin mRNA before the mixture was digested with RNase and protected fragments separated on a polyacrylamide gel. In D, 40 μg of adult rat brain RNA was used. **A:** A representative experiment using a probe specific for type II Na⁺ channel mRNA and corresponding to sequences in the 5′ untranslated and coding regions. The predicted size of the fragment protected by the 219-bp probe (shown in lane Na) was 128 bp; the predicted size of the fragment protected by the 62-bp cyclophilin probe (shown in lane Cy) was 37 bp. The sizes of the probes and protected fragments were estimated using an adjacent sequencing ladder and molecular size markers (data not shown), which were used to determine the position of the molecular sizes indicated on the right. Cyclophilin probe was not added to the brain and liver RNA samples. **B–D:** Similar experiments were done using other type-specific Na⁺ channel mRNA probes. Only the portion of the gel containing the protected fragments of Na⁺ channel mRNA is shown: (B) a 149-bp probe specific for type II Na⁺ channel mRNA and corresponding to sequences in the 3′ untranslated region protected a 76-bp fragment, (C) a 149-bp probe specific for type I Na⁺ channel mRNA and corresponding to sequences in the 3′ untranslated region protected a 60-bp fragment, and (D) a 157-bp probe specific for type III Na⁺ channel mRNA and corresponding to sequences in the 3′ untranslated region protected an 80-bp fragment.

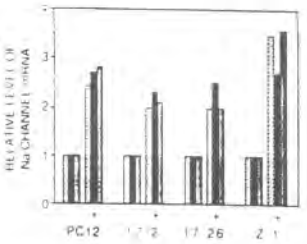

FIG. 3. Induction of Na^+ channel mRNA expression in untreated and NGF-treated PC12, MM17-2, MM17-26, and Z-1 cells. Na^+ channel mRNA, as detected by northern blot analysis (striped), RNase protection assay with a 5′-directed probe (solid), or RNase protection assay with a 3′-directed probe (gray), was quantified by densitometry, and for each cell line, the levels of Na^+ channel mRNA in NGF-treated cells (+) are plotted relative to that of Na^+ channel mRNA in untreated cells (−).

Electrophysiological recording and analysis

Na^+ current density was calculated as previously described (Ginty et al., 1992), using measurements of peak Na^+ current and cell membrane capacitance (as an indication of membrane area) obtained during whole-cell patch clamp recordings. Statistical significance was determined using a two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Northern blot analysis (Fig. 1) was used to determine if NGF increased the level of Na^+ channel mRNA in PC12 sublines (MM17-2, MM17-26) stably overexpressing c-Ha-*ras*(Asn-17). As in previous studies (Ginty et al., 1992), Na^+ channel mRNA was detected using a probe corresponding to a region highly conserved among Na^+ channel genes, and the signal was normalized to the signal corresponding to cyclophilin mRNA, which is constitutively expressed in PC12 cells and unaffected by NGF (Machida et al., 1989). In MM17-2 and MM17-26 cells treated with NGF, *ras*-dependent events such as transin gene expression (Fig. 1) and neurite outgrowth did not occur, consistent with our previous studies of these cells (Szeberenyi et al., 1990). In contrast, 2.0–2.5-fold increases in Na^+ channel mRNA were consistently detected in NGF-treated MM17-2 and MM17-26 cells, similar to the 2.5-fold increases observed in the parental PC12 cells and comparable to increases in parental PC12 cells stably transfected with a gene conferring neomycin resistance (Z-1), which served as a control for any effects associated with stable transfection of PC12 cells or with the maintenance of the stably transfected PC12 cell lines in culture medium containing the antibiotic neomycin.

RNase protection assays were used to determine if expression of any of the identified Na^+ channel genes expressed in the nervous system could account for the NGF-mediated increase in Na^+ channel mRNA in MM17-2 and MM17-26 cells. Probes specific for the type I, II, IIa, and III Na^+ channel mRNAs were generated from unique sequences in the 3′ and 5′ untranslated regions of the Na^+ channel genes (see Materials and Methods). Samples of total cellular RNA were hybridized with a mixture containing one of these probes and a probe specific for cyclophilin mRNA, allowing the Na^+ channel mRNA signal to be normalized to the cyclophilin mRNA signal. Type II Na^+ channel mRNA was detected in RNA from PC12, MM17-2, MM17-26, and Z-1 cells with either a 5′-directed probe (Fig. 2A) or a 3′-directed probe (Fig. 2B). In contrast, when a type I-specific probe (Fig. 2C) or a type III-specific probe (Fig. 2D) was used, transcripts were detected in RNA from rat brain, as expected (Beckh et al., 1989), but were not detected in RNA from PC12, MM17-2, MM17-26, or Z-1 cells, even when gels were exposed to film for 72–96 h (data not shown). Type IIa mRNA was also not detected in any of the PC12 cell lines (data not shown). Although there was some clonal variability in the basal levels of Na^+ channel mRNA in the sublines, based on densitometric analysis, the increases in type II Na^+ channel mRNA in NGF-treated MM17-2 and MM17-26 cells (2.3–2.5-fold) were similar to that observed in NGF-treated PC12 and Z-1 cells (2.7-fold). The results are in accord with the northern blot analysis (Fig. 3) and, in addition to providing evidence that the NGF-mediated increase in Na^+ channel mRNA is independent of *ras* activity, further suggest that the increase in Na^+ channel mRNA in MM17-2 and MM17-26 cells treated with NGF for 7 days can be accounted for by an increase in type II Na^+ channel mRNA.

Because the level of functional Na^+ channel expression

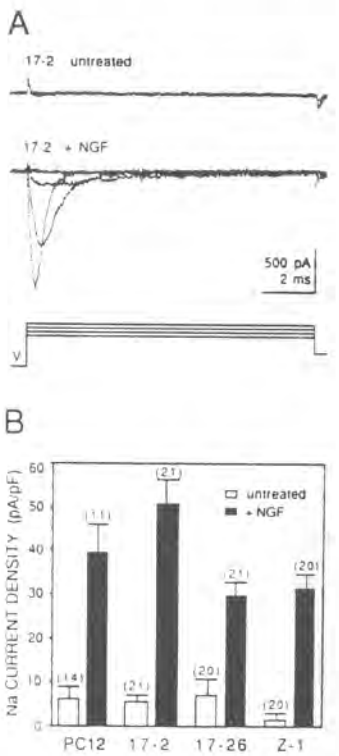

FIG. 4. Electrophysiological analysis of functional Na^+ channel expression. **A:** Representative recordings of voltage-dependent Na^+ currents in untreated and NGF-treated MM17-2 cells. Superimposed current records illustrate the response to depolarizing pulses to −40, −30, −20, and −10 mV, from a prepulse potential of −120 mV and holding potential of −80 mV. **B:** Average Na^+ current density in untreated and NGF-treated PC12, MM17-2, MM17-26, and Z-1 cells. Data are mean ± SEM (bars); number of cells in each group is in parentheses. Cell membrane capacitance (mean ± SEM; in pF) for untreated and NGF-treated cells, respectively, was as follows: PC12 cells, 8.5 ± 0.4 and 20.4 ± 0.9; MM17-2 cells, 8.0 ± 0.5 and 11.4 ± 0.7; MM17-26 cells, 7.1 ± 0.8 and 11.8 ± 1.2; and Z-1 cells, 6.3 ± 0.4 and 16.5 ± 0.8.

appears to be influenced not only by changes in gene expression, but also by translational and posttranslational events as well (Scheinman et al., 1989; Ginty et al., 1992), whole-cell patch clamp recordings were used to determine if NGF increased functional Na^+ current density in MM17-2 and MM17-26 cells. NGF caused a dramatic increase in the magnitude of the Na^+ currents that could be elicited in the MM17-2 and MM17-26 cells (Fig. 4A). NGF also increased cell membrane capacitance (and presumably cell size), although the change was smaller than we observed for the parental PC12 cell lines (see Fig. 4 legend), consistent with the neurite outgrowth we observed in the parental PC12 cells and the lack of neurite outgrowth in the MM17-2 and MM17-26 cells. Despite the increases in cell size, NGF significantly ($p < 0.005$) increased functional Na^+ current density in MM17-2 and MM17-26 cells, comparable to the increase we observed in PC12 and Z-1 cells (Fig. 4B).

Our results further define the role of *ras* activity in the induction of Na^+ channel expression by NGF and provide strong evidence for the importance of *ras*-independent mechanisms in NGF-mediated differentiation. Our results suggest that *ras* activity is not necessary for the NGF-induced increase in Na^+ channel mRNA or Na^+ current density, and in this regard Na^+ channel induction resembles other *ras*-independent responses to NGF, including survival in serum-free medium (Borasio et al., 1993) and activation of phospholipase C (Szeberenyi et al., 1992). In contrast, the relative insensitivity of NGF-mediated Na^+ channel induction to *ras* inhibition differs dramatically from NGF-induced MAPK, Raf-1, and RSK activity, SCG10 and transin gene expression, and neurite outgrowth, all which require *ras* activity and are sensitive to its inhibition (Szeberenyi et al., 1990; Kremer et al., 1991; Wood et al., 1992). Further analysis of the mechanisms underlying the induction of Na^+ channel expression by NGF should provide an avenue for determining the nature of these differences and in doing so help identify the molecular pathways responsible for NGF-induced neuronal differentiation.

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Minireview

Cellular components of nerve growth factor signaling

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Key words: Signal transduction; Nerve growth factor; PC12 cell; Neuronal differentiation; Ras proto-oncogene function

Contents

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Abbreviations: AP-1, activator protein-1; CaM-PK III, calmodulin-dependent protein kinase III; CRE, cAMP response element; CREB protein, cAMP response element binding protein; DAG, diacylglycerol; dbcAMP, dibutyryl-cyclic AMP; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GAP, GTPase activating protein; GRF, guanine-nucleotide releasing factor; HNGFR, high affinity nerve growth factor receptor; IP_3, inositol-triphosphate; LNGFR, low affinity nerve growth factor receptor; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK, MAP kinase/ERK-acivating kinase; MEKK, MAP kinase/ERK-activating kinase kinase; NF1, neurofibromatosis 1; NGF, nerve growth factor; NGFR, nerve growth factor receptor; PC-PLC, phosphatidylcholine-specific phospholipase C; PDGF, platelet-derived growth factor; PI3-K, phosphatidylinositol-3 kinase; PI3-P, phosphatidylinositol-3 phosphate; PK A, protein kinase A; PK C, protein kinase C; PK N, protein kinase N; PLCγ1, phospholipase Cγ1; PS/TK, protein serine/threonine kinase; PTK, protein tyrosine kinase; Rsk, ribosomal S6 kinase; RSV, Rous sarcoma virus; SH1, SH2, SH3 domains, Src homology 1, 2 and 3 domains; SNT, suc-associated neurotrophic factor-induced tyrosine-phosphorylated target; SRE, serum response element; SRF, serum response factor; TPA, tetradecanoyl phorbol acetate; TRE, TPA response element.

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1. Introduction

Polypeptide growth factors represent a large family of proteins that are implicated in the regulation of proliferation and differentiation of a great variety of cells (for a recent review see [1]). The common feature of these growth factors is that they recognize and bind to cell surface receptors whose activation triggers transcytoplasmic signal transduction events leading to altered gene expression and ultimately results in the mitogenic or differentiation response characteristic of the growth factor-target cell system. Signals are relayed partly through the generation of second messengers that can reach their target proteins by diffusing in the cell membrane (e.g., diacylglycerol) or in the cytosol (e.g., cyclic AMP, Ca^{2+}). Another, and most likely even more important, mechanism of signal transduction has been in the focus of scientific interest in the last few years. This mechanism is characterized by signal-induced, highly specific association of signaling proteins. In addition, activation of signal transduction routes often leads to redistribution of certain proteins in different compartments (e.g., membranes, nucleus) of the cell. Chemical modification (usually phosphorylation) of key signaling proteins can be induced by both second messengers and protein-protein interactions. The earliest cellular event of growth factor stimulation is the phosphorylation of specific proteins on tyrosine residues either by the activated receptor protein tyrosine kinase (PTK) itself or by intracellular non-receptor tyrosine protein kinases. This rush of tyrosine phosphorylation in turn will be converted into phosphorylation of another set of target proteins on serine and threonine residues. Protein serine/threonine kinases (P S/TKs) are coupled to tyrosine kinases by guanine nucleotide binding Ras proteins; the details of these interactions are unfolding at an unprecedented pace. Targets of S/TPKs include transcription factors whose altered state of phosphorylation induces a set of genes just after minutes of growth factor stimulation. Products of many of these early-response genes are transcription factors themselves and are believed to modulate the expression of secondary-response genes. The combined effect of the phosphorylation of pre-existing proteins and the appearance of a set of newly synthesized proteins are thought to produce the growth factor and cell-type specific phenotypic response. The family of growth factors whose signal transduction events follow the general mechanism described above include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF) and several other growth factors.

NGF, a member of the subfamily of neurotrophins, was the first growth factor to be discovered [2]. It is essential for the survival and differentiation of certain neurons of the peripheral and central nervous system [2]. The investigation of NGF-signaling was greatly facilitated and stimulated by the isolation and characterization of the rat PC12 pheochromocytoma cell line [3]. PC12 cells not requiring the presence of NGF to survive, while undergoing neuronal differentiation upon NGF treatment present themselves as ideal subjects to study NGF-signaling. The purpose of this review is to briefly summarize recent developments in PC12 research and to try to delineate signal transduction pathways that convey the effects of NGF in this model system. Our approach is intended to be functional emphasizing possible connections between signaling molecules and pathways (schematically summarized in Fig. 1.). The role of Ras proteins, key players in relaying receptor-originated signals to the cytoplasmic network of protein kinas . will be :scussed in some detail. For details of the biochemical and genetic aspects of NGF-signaling the reader should refer to excellent general review articles [4,5], as well as reviews dealing with individual components of signal transduction (listed in the relevant Sections).

2. Components of signal transduction in PC12 cells

2.1. The NGF receptor

2.1.1. $p75^{NGFR}$ and $p140^{trk}$

Two classes of cell surface NGF-binding sites have been identified: high affinity ($K_d = 10^{-10} - 10^{-11}$ M) and low affinity ($K_d = 10^{-9}$ M) receptor sites (for recent reviews see [6–9]). The nature of these receptors (designated HNGFR and LNGFR, respectively) has been a subject of controversy and the debate has still not been settled. Two transmembrane proteins with the ability to bind NGF have been identified and characterized. $p75^{NGFR}$ [10,11] is a cell surface glycoprotein lacking known catalytic activity, is expressed in all NGF-responsive cells [12–15], as well as in several non-neuronal cell types that do not respond to NGF [16]. It binds NGF with a low affinity. The role of $p75^{NGFR}$ is still not known, but it may be involved in NGF-signaling as a component of a high affinity NGFR (see below), function as a presentation receptor for NGF, a discriminator among various neurotrophins or as a recruiter of specific signaling proteins (reviewed in [7]). Recent evidence implicates $p75^{NGFR}$ in the process of apoptotic cell death of PC12 cells [17]. $p140^{trk}$ was discovered as the protooncogenic counterpart of a 70 kDa cellular oncoprotein identified in human colon cancer [18]. The *trk* oncogene emerged from the fusion of sequences from a protein tyrosine kinase gene (c-*trk*) and the tropomyosin gene. $p140^{trk}$ is a transmembrane glycoprotein that belongs to the family of receptor protein tyrosine kinases. Upon NGF binding [19–23] $p140^{trk}$ undergoes tyrosine phosphorylation and dimerization [24,25], a process characteristic of receptor PTKs [26]. $p140^{trk}$ is expressed at significant levels in NGF responsive cells of the nervous system only [7,9] and is required for the biological effect of NGF [27,28]. $p140^{trk}$ is a member of a family of neurotrophin receptors that also include $p145^{trkB}$ and $p145^{trkC}$, receptors for brain-devied neurotrophic factor and neurotrophin-3, respectively [7–9].

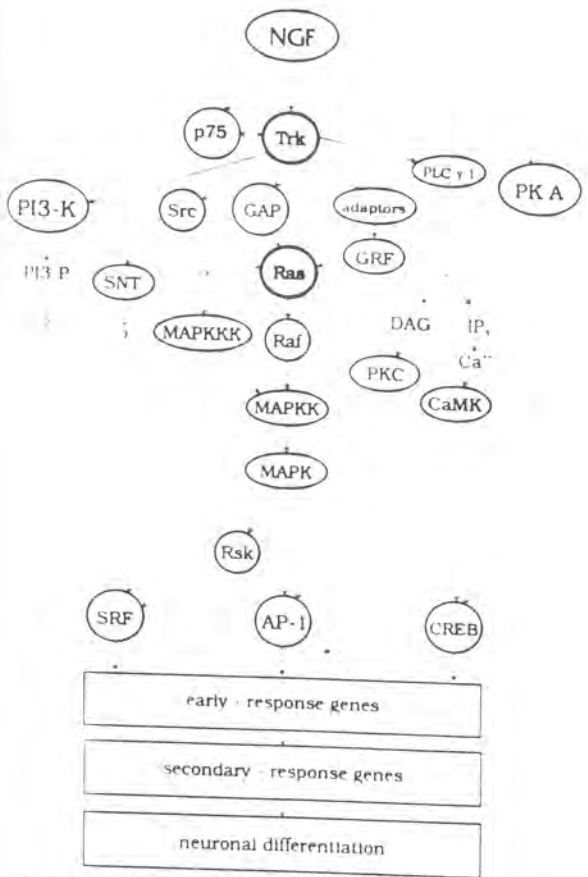

Fig. 1. Elements of nerve growth factor signaling in PC12 cells. Bold letters indicate components whose indispensable function in NGF signaling is firmly established. Arrows with solid and broken lines represent connections between signaling elements that are strongly supported by experimental evidence and largely hypothetical, respectively. Abbreviations: see footnote.

2.1.2. Two models

Two hypotheses have been formulated to describe the role of $p75^{NGFR}$ and $p140^{trk}$ in the formation of biologically active high affinity NGF receptors. According to the *p75 / Trk heterodimer model* [23], high affinity NGF binding sites are formed by the association of $p75^{NGFR}$ and $p140^{trk}$ molecules. Most of the evidence supporting this model comes from studies on NR18 cells, a mutant PC12 cell line expressing an altered, nonfunctional $p75^{NGFR}$ protein. These cells do not express HNGFR, and are resistant to NGF induction of differentiation, protein tyrosine phosphorylation and c-*fos* activation [27]. Expression of wild-type $p75^{NGFR}$ in these cells, however, generates high affinity NGF binding and partially restores their NGF-responsiveness [23,27,29,30]. Similarly, expression of high affinity NGF-binding sites in COS-1 cells requires the coexpression of $p75^{NGFR}$ and $p140^{trk}$ [23]. Additional support for the significance of $p75^{NGFR}$ comes from experiments using a chimeric receptor consisting of the extracellular domain of the EGF-receptor and the transmembrane and intracellular domains of $p75^{NGFR}$. This receptor is able to convey EGF-induced differentiation in PC12 cells [31]. These data are consistent with a model in which $p75^{NGFR}$, presumably in a complex with $p140^{trk}$, plays an important role in transmembrane NGF signaling.

In contrast, the *Trk homodimer model* [19,22,25] is based on the assumption that dimerization of p^{140trk} molecules upon binding of NGF produces high affinity NGF binding sites even in the absence of $p75^{NGFR}$. Several lines of evidence support this hypothesis. NIH 3T3 cells ectopically expressing $p140^{trk}$ (but not $p75^{NGFR}$) display high affinity NGF binding sites [19], can be mitogenically stimulated and transformed by

NGF and show NGF-induced activation of the c-*fos* gene [22,25]. Similar results were obtained using *Xenopus* oocytes transfected with *trk* cDNA: maturation of these cells was observed upon treatment with NGF [21]. Anti-$p75^{NGFR}$ antibodies able to interfere with NGF-p75 binding do not inhibit NGF-stimulated survival or differentiation of PC12 cells [32]. Mutant NGF molecules that lost their ability to bind to $p75^{NGFR}$ but not to $p140^{trk}$ are capable of inducing neuronal differentiation of PC12 cells [33]. In addition, no evidence has so far been presented to demonstrate physical association between $p75^{NGFR}$ and $p140^{trk}$ [8]. Finally, mice carrying disrupted $p75^{NGFR}$ genes have virtually normal development of sympathetic ganglia [34]. These observations raise serious doubts regarding a significant role of $p75^{NGFR}$ in NGF signaling. However, since some of the data are conflicting, a final decision regarding the two models awaits further experiments. It should be emphasized, though, that a paramount role of the $p140^{trk}$ proto-oncogenic protein in transmembrane signaling by NGF is questioned by neither of these models.

2.1.3. Possible intracellular targets of the NGF receptor

The relay of signals by receptor PTKs across the cell membrane is accomplished by the phosphorylation of intracellular target proteins on tyrosine residues upon binding of the ligand to the receptor. In addition, proteins containing src homology 2 (SH2) domains are able to physically interact with the activated receptor (reviewed in [26]) by binding to phosphotyrosine containing sequences in the receptor. NGF treatment of PC12 cells is followed by rapid tyrosine-phosphorylation of a distinct set of proteins [35], including proteins that are capable of signal transduction by generating second messengers or by association with downstream effectors. The first identified substrate of $p140^{trk}$ was phospholipase Cγ1 (PLCγ1). This enzyme is rapidly tyrosine phosphorylated [36,37] and activated [36] upon NGF treatment of PC12 cells generating the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-biphosphate. In vitro phosphorylation assays using cell extracts from NGF-treated cells indicate that the enzyme responsible for PLCγ1 phosphorylation is $p140^{trk}$ or another, tightly associated PTK [37]. Phosphatidylinositol 3-kinase (PI3-K), an enzyme that produces the putative second messenger phosphatidylinositol 3-phosphates, is also tyrosine phosphorylated and activated after minutes of NGF treatment [38–40]. Both PLCγ1 and PI3-K contain SH2 domains and are physically associated with the NGF-activated $p140^{trk}$ protein [37,39]. Another possible target of $p140^{trk}$ kinase has recently been described: SNT (for *s*uc-associated *n*eurotrophic factor-induced *t*yrosine-phosphorylated target), a putative signal transducing protein, is rapidly tyrosine-phosphorylated in PC12 cells treated with NGF. Phosphorylation of SNT appears to correlate with neuronal differentiation, but its role in the process is not clear [41]. Other possible effectors of the NGF receptor are GTPase activating proteins (GAPs) and guanine-nucleotide releasing factors (GRFs).The possible role of these proteins in NGF signaling will be discussed in connection with Ras proteins (see below). Association of the mitogen-activated protein kinase ERK1 with the activated $p140^{trk}$ protein has recently been reported [42] and will be discussed later.

2.2. Nonreceptor protein tyrosine kinases

These proteins represent a rapidly growing family of cytoplasmic PTKs with potential involvement in diverse signal transduction processes (for recent reviews see [43,44]). Common structural features of members of this family include the absence of an extracellular domain and the presence of a catalytic domain (Src homology 1 or SH1 region). Most of these proteins contain SH2 and SH3 domains as well that enable them to associate with tyrosine-phosphorylated or proline-rich protein regions, respectively [45]. Nonreceptor PTKs are believed to relay signals from cell surface receptors lacking tyrosine kinase activity to intracellular target proteins.

c-Src, the proto-oncogenic counterpart of the Rous sarcoma virus (RSV) oncoprotein is strongly implicated in NGF signaling. A highly active modified Src protein is strongly expressed in neurons [46]. Infection of PC12 cells with RSV induces neurite outgrowth [47], while microinjection of these cells with anti-Src antibodies blocks NGF and FGF, but not activated Ras-stimulated neuritogenesis. In turn, v-Src-induced neuronal differentiation is inhibited by anti-Ras antibodies or expression of a dominant negative Ras protein [48]. In addition, activated Src induces some of the early and secondary response genes in a Ras-dependent manner [49]. These observations place c-Src between the NGF receptor and Ras as an important mediator in NGF signaling. The nature of contacts of c-Src with its upstream regulators and downstream effectors, however, remains to be clarified.

2.3. Ras proteins

2.3.1. General features of Ras proteins

ras genes represent a ubiquitous family of proto-oncogenes found in all eukaryotes studied so far. The products of the three mammalian *ras* genes, the highly conserved H-Ras, K-Ras and N-Ras proteins are small, membrane-associated polypeptides (designated p21 proteins), whose structural and functional characteristics have been discussed in detail in several recent reviews [50–59]. These proteins have been in the focus

of intensive scientific attention during the last 15 years for two reasons. First, *ras* genes are frequently activated mutationally in a number of human tumors. Second, Ras proteins, belonging to the class of guanine-nucleotide binding proteins which alternate between an inactive, GDP-bound and an active, GTP-bound state, therefore may function as molecular switches in a variety of signaling systems. By coupling cell surface receptors to intracellular protein kinases they serve as mediators of diverse biological responses, e.g., proliferation and transformation of fibroblasts, activation of T cells, differentiation of hematopoietic and neuronal cells, mesoderm induction [52,58,60,61].

2.3.2. Ras and NGF signaling

A central role of Ras in signal transduction from the NGF receptor of PC12 cells is supported by several lines of independent evidence. First, expression of constitutively active Ras proteins leads to morphological differentiation of PC12 cells [62–67]. Second, microinjection of PC12 cells with anti-Ras antibodies inhibits NGF-induced neuritogenesis [68]. Third, similar inhibition can be achieved in PC12 cells expressing a dominant inhibitory Ras protein [69]. The mutant p21 protein (designated Ha-Ras Asn-17) displays a preferential GDP binding activity [70], and intereferes with the function of endogenous Ras proteins [71] by competing for a guanine-nucleotide releasing factor ([72], and see below), thereby inhibiting the proliferation and mitogenic response of NIH 3T3 cells [73]. With the help of the Ha-*ras* Asn-17 gene not only the requirement for Ras function in PC12 cell differentiation was confirmed, but NGF signaling pathways requiring different levels of Ras activity have also been identified (see Section 3). Finally, measurement of the ratio of Ras GDP/Ras GTP in PC12 cell extracts provided direct evidence for the rapid and sustained activation of Ras proteins upon treatment with NGF [74–77]. These observations left no doubt that Ras proteins were crucial players in signal transduction from the NGF receptor, but the way signals are relayed by them remained obscure.

2.3.3. Upstream and downstream connections of Ras

During the last two years intensive efforts by several laboratories led to enormous advances in our understanding both the regulation and effector functions of Ras proteins.

The functional state of Ras proteins is modulated by two types of regulatory proteins. GTPase activating proteins (Ras-GAP and the product of the neurofibromatosis 1 gene, NF1) stimulate the intrinsic GTPase activity of proto-oncogenic Ras leading to conversion of the active, Ras-GTP complex to the inactive Ras-GDP form. Besides this downregulator function, GAPs might act as effectors for Ras as well (reviewed in [78,79]). Guanine-nucleotide releasing factors (GRFs) activate Ras proteins by stimulating the substitution of bound GDP for GTP. Several mammalian GRFs have recently been identified, some with ubiquitous (e.g., Sos proteins homologous to the Son of sevenless protein in *Drosophila*), others with more restricted expression (e.g., Vav in T cells, p140 Ras GRF in brain; reviewed in [80–83]).

Both types of Ras regulators can be coupled to activated PTK receptors. Direct association of GAP proteins with the activated PDGF receptor is well documented [26,83]. Moderate stimulation of Ras-GAP (but not NF1) activity has been observed in NGF-treated PC12 cells, without physical association of GAP and Trk [76]. The functional significance of this finding is not clear, and the possible Ras-regulator/Ras-effector role of GAPs in NGF signaling needs to be further analyzed.

The mechanisms of coupling PTK receptors to Ras proteins via GRFs has been described in a series of papers by several laboratories during the last year (see references in [80,82]). An evolutionally conserved set of small proteins consisting of SH2 and SH3 domains have been identified in nematode, *Drosophila* and mammalian cells. These proteins are capable of binding to tyrosine kinase receptors and specific target proteins via their SH2 and SH3 domains, respectively. Such an adaptor protein, designated Grb2, has been found to be expressed in a wide variety of mammalian tissues. It binds to the activated EGF receptor in NIH 3T3 cells, to other adaptors (e.g., proteins called Shc), and, most importantly, to the guanine-nucleotide release factor Sos. In addition, EGF activation of Ras was found to be dependent on the expression of Grb2, and microinjection of Grb2 and Ha-Ras proteins induced DNA synthesis in fibroblasts. These observations established Grb2 as an important component of mitogenic signal transduction between receptor PTKs and Ras. A similar mechanism might function in NGF signaling. Shc proteins become tyrosine phosphorylated and complexed with Grb2 in NGF-treated PC12 cells [84,85]. In addition, overexpression of Shc proteins induces neurite outgrowth in a Ras-dependent manner [84]. Activated NGFR, unlike EGFR, hovewer, does not bind Grb2 protein [85]. Thus, although the general structure of the receptor-adaptor-GRF-Ras chain might be similar for both receptors, NGFR may use an adaptor other than Grb2. This assumption is supported by the report that Crk, another member of the SH2-SH3 adaptor family, is able to induce neurite formation in PC12 cells in a Ras-dependent manner [86]. The pace of recent developments in this field promises a rapid understanding of Ras regulation in PC12 cells.

Despite enormous efforts by many researchers to identify downstream targets of Ras proteins, little advance had been achieved in this field until very re-

cently. Adenylate cyclase, a major effector of RAS in *Saccharomyces cerevisiae*, is most likely not a target of Ras in mammalian cells [87]. Phosphatidylinositol-specific PLCτ, another strong candidate, was found to be regulated by growth factors in a Ras-independent manner in both NIH 3T3 [88] and PC12 cells [89]. Phosphatidylcholine-specific phospholipase C (PC-PLC) is a Ras target in fibroblasts, but its role and relationship to Ras in PC12 cells remains to be determined [90,91]. Phospholipase A_2 (PLA_2), an enzyme generating arachidonic acid- and lysophospholipid-derived putative second messengers, has been implicated in Ras-signaling, but the available evidence supporting this idea is still scarce and circumstantial [52]. Protein kinase C, activated by the PLCτ-product diacylglycerol, appears to be controlled by Ras in certain cell types [52], but not in others. Latterly, a major signaling pathway has recently been identified as a downstream target for Ras: a protein serine/threonine kinase cascade with proto-oncogenic Raf proteins as the most proximal components of this chain of signal transduction proteins (see below).

2.4. Protein serine / threonine kinases

Transduction of receptor PTK-generated signals across the cytoplasm to the nucleus is mediated by a large and still increasing family of PS/TKs [92], both in mitogen-treated fibroblasts and in NGF-differentiated PC12 cells. A cascade of protein kinases has emerged recently from this tentatively defined mass of PS/TKs as the major mitogenic and, most likely, NGF signaling pathway. The outstanding significance of this signaling route is strongly supported by its conservation during eukaryotic evolution. The cascade consists of Raf kinases, mitogen-activated protein kinases (MAPKs) and their activators, ribosomal S6 kinase (Rsk) and possibly other PS/TKs whose ultimate substrates include important regulatory nuclear proteins (for recent reviews see [93–100]).

2.4.1. The Raf family

General properties of Raf proteins. This family of PS/TKs consists of three related cytosolic proteins (reviewed in [93,94]). c-Raf-1, A-Raf and B-Raf have a similar overall domain-structure: they share three highly conserved domains, CR_1, CR_2, and CR_3. The N-terminal cysteine rich CR_1 acts as a negative regulatory domain; its truncation renders Raf proteins constitutively active. CR_2 becomes phosphorylated during the mitogenic response, thereby playing a positive regulatory role; CR_3 is the catalytic kinase domain. c-Raf-1, the cellular counterpart of the retroviral oncoprotein v-Raf, is characterized by a ubiquitous expression indicating a role in housekeeping metabolism. The other two members of the family have a more restricted pattern of expression: A-Raf is produced in large quantities in the cells of the urogenital tract, while B-Raf is primarily expressed in the central nervous system and in the gonads.

c-Raf-1 and mitogenesis. c-Raf-1 is activated by phosphorylation on tyrosine and serine residues in mitogen-treated cells [93,94] and is subsequently translocated into the nucleus [93]. Furthermore, hyperphosphorylation of c-Raf-1, that can be easily detected by a visible shift of the electrophoretic mobility of the protein, is usually accompanied by an increase in its protein kinase activity. All strong mitogens tested are capable of activating c-Raf-1. In addition, physical association of c-Raf-1 to the activated PDGF and EGF receptors have been reported; binding of c-Raf-1 to other receptors, such as the receptors for insulin, CSF or NGF, has not been detected [93], indicating receptor-specific differences in its mode of activation. The most direct evidence for the involvement of c-Raf-1 in mitogenic signal transduction came from experiments using the expression of antisense c-Raf-1 RNA or dominant inhibitory dead-kinase c-Raf-1 mutants in fibroblasts. The results of these studies indicate that c-Raf-1 function is absolutely required for the proliferation as well as mitogenic and gene activation response of these cells [101–103].

c-Raf-1 and B-Raf in PC12 cell differentiation. PC12 cells constitutively express high levels of the c-Raf-1 and B-Raf proteins [104–106]. As in fibroblasts, a number of growth factors (NGF, FGF, insulin, EGF) and second messenger analogs (TPA, Ca^{2+}) induce c-Raf-1 hyperphosphorylation [88,107]. Similarly, treatment of PC12 cells with NGF, EGF, FGF or TPA, stimulates phosphorylation of the B-Raf protein [88,105]. Moreover, NGF-induced hyperphosphorylation of both c-Raf-1 and B-Raf is accompanied by the stimulation of their kinase activity [88]. Expression of v-Raf [88] or activated c-Raf-1 [108] induces neurite formation and the activation of NGF-regulated genes [108]. These observations strongly suggest that Raf proteins are important components of the NGF signaling machinery, and that, in contrast to the role of c-Raf in the mitogenic response of fibroblasts, they appear to be members of the major signal transduction route mediating neuronal differentiation in PC12 cells.

The Ras-Raf connection. As mentioned earlier, members of the Raf family of protein kinases are believed to act as downstream effectors of Ras proteins in signal transduction events of a number of experimental systems, including *Drosophila*, *C. elegans*, *Xenopus*, and mammalian cells (see references in [109]). Recent observations indicate that this might be the case in NGF-signaling as well. Hyperphosphorylation of c-Raf-1 can be detected in PC12 cells expressing activated Ras [107]. Hyperphosphorylation and activation of c-Raf-1 and B-Raf proteins induced by NGF

treatment can be blocked by the interfering Ras mutant Ha-Ras Asn-17 [88,107]. In contrast, neurite formation induced by expression of v-Raf in PC12 cells does not require Ras function [88]. Although the exact biochemical mechanisms by which Ras couples Raf proteins to the activated NGF receptor is not known, these observations clearly position Raf downstream of Ras in NGF signaling.

Most recently, direct association of Ras and c-Raf-1 proteins have been demonstrated in several laboratories. In vitro binding of Ras in the active conformation to the N-terminus of c-Raf-1 was detected by using immobilized proteins [110,111] or coimmunoprecipitation by an anti-Raf antibody [112]. The latter approach provided evidence for Ras-Raf association in PC12 cell extracts as well [112]. Functionally effective binding of Ras and Raf was also demonstrated by the elegant in vivo two hybrid system [109,113]. Ras-Raf complexes attract other proteins as well (e.g., mitogen-activated protein kinase kinase, see below) and might function as parts of multiprotein signaling units [110,113].

2.4.2. *Mitogen-activated protein kinases and their activators*

MAPKS and the mitogenic response. The group of mitogen-activated protein kinases (MAPKs, also known as extracellular signal-regulated kinases, ERKs) function as *switch kinases*: they undergo tyrosine and threonine phosphorylation and convert the activating tyrosine phosphorylation into a downstream cascade of serine / threonine phosphorylation [95–100]. At least two members of this family of closely related isoforms, $p44^{mapk/erk1}$ and $p42^{mapk/erk2}$, are targets of multiple mitogenic pathways. The mitogenic response of fibroblasts to growth factors requires a rapid, sustained Ras-dependent stimulation of MAPKs, that leads to the nuclear translocation of a fraction of these proteins. MAPKs are also activated by transforming Ras and Raf proteins, supporting the notion that these enzymes are integral parts of the major signal transduction pathway originating from the growth factor receptors. The ultimate evidence for the requirement of MAPK function in mitogenic signaling was recently presented by experiments using dominant negative $p44^{mapk/erk1}$ mutants: fibroblasts expressing the mutant proteins show reduced cell growth and growth factor-induced gene activation [114].

MAPKs and NGF signaling. Continuous NGF treatment of PC12 cells induces rapid tyrosine phosphorylation, activation and nuclear translocation of MAPKs [107,115–117]. These effects are dependent on the expression of $p140^{trk}$ protein, but do not require $p75^{NGFR}$ [104]. In addition, MAPK activation can be induced by expressing oncogenic Ras proteins in PC12 cells [118], while expression of the dominant negative Ha-Ras Asn-17 protein blocks NGF stimulation of MAPKs [107,115,117]. Growth factor-induced association of $p42^{mapk}$ with $p140^{trk}$ NGF receptor was also observed. Interestingly and somewhat mysteriously, this binding does not depend on the activation of the NGF receptor by its ligand; it can be induced by EGF or FGF treatment of PC12 cells as well [119]. Another unexpected finding was that expression of an oncogenic form of c-Raf-1 failed to activate $p44^{mapk}$ in PC12 cells. This observation can be explained by the existence of Raf-independent signals that can synergize with the Raf-mediated pathway to fully activate MAPKs [107]. Understanding early events in neuronal differentiation will require dissection and identification of such parallel routes. Nevertheless, MAPKs are strongly implicated in NGF signaling, although direct evidence supporting their role, to be obtained by exploiting the dominant negative MAPK mutant system (see above), remains to be presented.

MAPK activators: MAPKKs and MAPKKKs. Although MAPKs are positioned distal to Raf proteins in transcytoplasmic signaling, they are not phosphorylated and activated directly by Raf kinases. At least two additional classes of protein kinases function as upstream regulators of MAPKs: MAPK kinases (MAPKKs, also known as MAP kinase / ERK-activating kinases or MEKs) and MAPK kinase kinases (MAPKKKs) (reviewed in [95,97–100]). MAPKKs are complexed with Ras and c-Raf-1 ([110,113]; see above). Upon activation by phosphorylation by c-Raf-1, MAPKKs act as dual specificity kinases [120]: they phosphorylate MAPKs on both tyrosine and threonine residues. Besides c-Raf-1, another MAPKKK is also capable of activating MAPKK. This enzyme, referred to as MEK kinase, (MEKK) is believed to mediate a c-Raf-1 independent signal. Although both MAPKKs [121] and MAPKKKs [122] have been identified and partially characterized in PC12 cells, the details of their involvement in NGF signaling remain to be determined.

MAPK substrates. Several proteins have been identified recently as putative substrates for MAPKs. $pp90^{rsk}$, a serine / threonine specific protein kinase [123] appears to be a major downstream target for the Ras-Raf-MAPK pathway (reviewed in [95–98,100]). $pp90^{rsk}$ was first isolated using its ability to phosphorylate ribosomal S6 protein. It is phosphorylated and activated in vitro by MAPK. Upon growth factor stimulation of cells both MAPKs and $pp90^{rsk}$ translocate into the nucleus, and are thought to phosphorylate key transcription factors. In PC12 cells NGF-induced phosphorylation and activation of MAPKs are accompained by phosphorylation and activation of $pp90^{rsk}$, as well as by the physical association of the two types of signaling proteins [124]. Activation of $pp90^{rsk}$ kinase activity by NGF treatment was found to be strongly dependent on Ras function [115]. These observations place $pp90^{rsk}$ to

the distal end of the NGFR → Ras → Raf → MAPK signaling chain.

Evidence suggesting a MAPK-target function for other proteins is much more circumstantial, and mostly relies on in vitro phosphorylation experiments. PLA_2, a putative signal transduction enzyme (see Section 2.3.3) is phosphorylated and activated by MAPK in vitro [124a]. $p62^{TCF}$, a transcription factor that binds to the serum response element (SRE) of the c-*fos* promoter is an in vitro substrate for MAPKs, and its affinity for SRE is increased by this phosphorylation [125]. The nuclear proto-oncoproteins c-Fos and c-Jun are also substrates for MAPKs in vitro [126], but the in vivo relevance of these observations is still a matter of debate. In PC12 cells, phosphorylation of c-Fos and Zif 268 (another early response gene product) upon NGF treatment is dependent on Ras function (Szeberényi and Cooper, unpublished), but no firm link has been established between MAPKs and these phosphorylation events.

2.4.3. Protein kinase A

The involvement of cyclic AMP (cAMP) and cAMP-dependent protein kinase (protein kinase A, PK A; for a recent review see [127]) isozymes in NGF-induced neuronal differentiation is still a matter of controversy. A transient increase in cAMP levels found in NGF-stimulated PC12 cells was not confirmed by other laboratories (see references in [5]). Membrane permeable cAMP derivatives can induce process formation in PC12 cells, but the neurites are much shorter than those induced by NGF [67]. PC12 cell lines deficient in PK A activity display normal NGF stimulation of neurite formation, c-*fos*, *zif 268*, ornithine decarboxylase and transin expression, as well as phosphorylation of several proteins [128–131]. Other effects of NGF, e.g., expression of sodium channels [131] and downregulation of calmodulin-dependent protein kinase III [132] are inhibited in PK A-deficient cells. These observations suggest that PK A is not a major player in NGF-induced neuronal differentiation, but may be involved in certain aspects of NGF signaling.

2.4.4. Protein kinase C

The name protein kinase C (PK C) covers a large family of PS/TKs, with at least nine members (reviewed in [133–135]). Several PK C isoenzymes can be activated by DAG and by phorbol esters (e.g., tetradecanoyl phorbol acetate, TPA). DAG can be generated by the stimulation of several phospholipid metabolizing enzymes: PLC_γ, PC-PLC and through a phosphatidic acid intermediate, phospholipase D (PLD) [133]. PK C isoenzymes widely expressed in various tissues, therefore, may act as convergence points of several upstream signaling routes regulating cell proliferation and differentiation [135].

Similarly to the situation with PK A, the significance of PK C in NGF signaling is still debated. PC12 cells express all known PK C isoforms [135]. Upon treatment of these cells with NGF, a rapid, small increase in PK C activity can be observed [136,137]. Direct stimulation of PK C by TPA treatment does not cause morphological differentiation of PC12 cells, but synergizes with dibutyryl-cAMP (dbcAMP) for process formation [67]. On the other hand, downregulation of PK C by prolonged treatment with TPA did not cause an inhibition of NGF-stimulated differentiation, early and secondary response gene expression [89,128,138]. Although other studies found a requirement of PK C activity for NGF-induced neurite formation [139] and gene activation [140], at present most of the experimental evidence supports only an accessory role for PK C in NGF signaling. This statement, however, may not apply for PK C isoforms insensitive to downregulation by phorbol esters (for details see [135]).

2.4.5. Other protein kinases

Several other PS/TKs are also affected by NGF treatment in PC12 cells whose function in the signal transduction process is even more obscure than that of PK A and PK C. Protein kinase N (PK N) is a relatively well characterized cytosolic enzyme distinct from other known protein kinases [141]. It is rapidly activated by NGF, and therefore might have a receptor-proximal position in NGF signaling. The recent extensive purification of the enzyme should speed up its further characterization [142].

Calmodulin-dependent protein kinase III (CaM-PK III) is downregulated by NGF treatment in a PK A-dependent manner [132]. Ribosomal S6 kinases (other than $pp90^{rsk}$) are activated in NGF-treated PC12 cells [143]. The significance of these and possibly other phosphorylation events in neuronal differentiation remains to be determined.

2.5. Gene expression

2.5.1. Gene activation in response to growth factors

The phosphorylation events evoked by the stimulation of mitogenic or differentiation signal transduction pathways affect the function of many cellular proteins. One class of these proteins, the transcription factors, has a paramount importance in the biological consequences of growth factor stimulation: their phosphorylation triggers a highly specific pattern of gene expression that will have a long-term impact on the phenotype of the cell. Expression of two major classes of genes is characteristic of the epigenetic response to growth factor treatment: induction of early-response genes is rapid and transient; it is mediated by the phosphorylation-dependent activation of pre-existing transcription factors. In contrast, expression of sec-

ondary-response genes is more delayed and sustained, and is dependent on de novo synthesis and activation of transcription factors, many of them products of early-response genes. Products of secondary-response genes, in turn, are believed to be directly involved in the shaping of the ultimate phenotypic effect of growth factors.

2.5.2. Linking cytoplasmic protein serine / threonine kinases to early-response gene expression

Expression of early-response genes is primarily controlled at the level of transcription initiation. The promoter regions of these genes contain common sequence elements recognized by specific transcription factors. Differential regulation of early-response genes is thought to be achieved by unique combinations of sequence elements in the individual promoter regions (for review see [144]). Binding of transcription factors to their cognate sequences and their ability to modulate transcription of the gene is mostly determined by their state of phosphorylation (for recent reviews see [125,144–147]). Several PS / TKs described in the preceding Section have been implicated in the phosphorylation and regulation of pre-existing transcription factors during growth factor signaling. The main promoter-targets of NGF-stimulated signal transduction pathways are the serum response element (SRE), cAMP response element (CRE), and TPA response element (TRE). The presence of all three elements in the c-*fos* promoter makes this proto-oncogene the prototypical NGF-inducible early-response gene [125,145].

SRE sequences are present in the promoters of many serum-inducible genes, and are believed to be the primary promoter elements of serum growth factor response [125]. SRE is recognized by and forms a ternary complex with a dimeric nuclear phosphoprotein (serum response factor, SRF) and an accessory protein (ternary complex factor, $p62^{TCF}$). SRF itself is an early-response gene product in PC12 cells: transcription of its gene is stimulated by NGF treatment in the absence of protein synthesis [148]. SRF and $p62^{TCF}$ are in vitro substrates for the PS / TKs Rsk and MAPKs, respectively. These kinases are known to be translocated into the nucleus upon activation. More importantly, SRE was found to be the genomic target of activated Src, Ras and Raf oncogenes [125]. All these observations serve as indirect, but strong evidence to support the notion that SRE-containing genes are linked to the distal end of the receptor-Src-Ras-Raf-MAPK-Rsk cascade.

TRE was discovered as a promoter element activated by phorbol ester (TPA) stimulation of a PKC-mediated pathway [145]. Subsequently, a wide array of receptor-binding ligands (polypeptide hormones, growth factors, cytokines, neurotransmitters) and activated oncoproteins (v-Src, Ha-Ras, v-Raf) have been found to stimulate gene expression via TRE. Activation of TRE is mediated by a dimeric transcription factor (activator protein-1, AP-1) consisting of the members of Fos (c-Fos, FosB, Fra1, Fra2) and Jun (c-Jun, JunB, JunD) protein families, capable of dimerizing through their leucine-zipper domains. Rapid induction of early-response genes through AP-1 is likely to be mediated by changes in the phosphorylation state of pre-existing c-Jun-homodimers. (Fos proteins are scarce in unstimulated cells.) c-Jun is activated by phosphorylation of serine residues in the N-terminal domain and by dephosphorylation of an inhibitory region near the C-terminus. Candidates for the catalysis of the former reaction include MAPKs [149] and a poorly characterized 'c-Jun kinase' [150]. Phosphorylation of these serine residues and consequent activation of c-Jun are stimulated not only by growth factor treatment, but by the expression of oncogenic Src, Ras and Raf proteins as well, indicating that the same major signal transduction pathway targets both SRE and TRE (see above; [151]). The activating dephosphorylation is believed to be performed by a putative PK C-regulated 'c-Jun phosphatase' [145]. Fos proteins contribute to AP-1 activity during the later phases of growth factor stimulation, when the Jun homodimers are transiently replaced by Jun-Fos heterodimers.

The regulation and role of AP-1 activity during NGF-induced neuronal differentiation is poorly understood. NGF stimulation of TRE is strongly Ras-dependent, but proceeds normally in PC12 cells downregulated for PK C by prolonged TPA treatment [89]. Increased phosphorylation of c-Fos in PC12 cells was observed after treatment with NGF, FGF, TPA [152] and EGF (J. Szeberényi and G.M. Cooper, unpublished data). An NGF-stimulated putative 'Fos-kinase' has been identified and partially purified [153]. Interestingly, phosphorylation of c-Jun in NGF-treated PC12 cells has not been reported yet. Thus, although TRE sequences are targets of both PK C-dependent and -independent pathways in PC12 cells, the exact nature and significance of AP-1 phosphorylation remain to be determined.

CRE sequences are present in the promoters of cAMP-regulated genes (for reviews see [145,154]). These elements are recognized by a family of related CRE-binding proteins (CREBs). Upon binding of cAMP to PK A, the catalytic subunits of the enzyme dissociate from its regulatory subunits and are translocated into the nucleus. Subsequently, CREB is activated by phosphorylation of a single serine residue by the PK A C-subunit. Depolarization in PC12 cells leads to the Ca^{2+}-dependent phosphorylation of the same serine in CREB [155]. CREB, therefore, appears to be a convergence point for cAMP-and Ca^{2+}-mediated signaling pathways in these cells [156,157]. Although neither cAMP, nor Ca^{2+} appear to play a substantial role

NGF-induced neuronal differentiation (see above). RE sequences might be crucial in the NGF-activation of certain genes [158].

2.5.3. Induction of early-response genes

As described earlier, a variety of agents, including growth factors, second messengers, neurotransmitters, etc. causes the rapid, protein synthesis-independent transcriptional activation of the so-called early-response genes in a number of tissues and cell lines (reviewed in [144,159]). Close to 20 of these genes code for verified or putative trancription factors [144], and thus are believed to act as 'third messengers' linking second messenger-regulated protein kinases to phenotype-related target genes. Most of the products of early-response genes, however, are poorly characterized, therefore, their role in nuclear signal transduction is yet to be substantiated. The behaviour of four of these genes (c-*fos*, c-*jun*, *zif 268*, *nur 77*) has been analyzed to some extent in NGF-treated PC12 cells, therefore, in this section we will concentrate on the properties, expression and posttranslational modification of these early-response gene products.

The proto-oncogene c-*fos* codes for a leucine-zipper protein that forms an active heterodimer (AP-1) with members of the c-Jun protein family (see above). The role of c-Fos protein as third messenger is still controversial and appears to be cell-type-dependent. Expression of anti-sense c-*fos* RNA in NIH 3T3 cells blocked *ras*- or *ras*-induced transformation of these cells [160,161]. In contrast, embryonal stem cells with homozygous knock-out mutations in their c-*fos* genes display apparently normal cell growth and differentiation [162]. c-*fos* is rapidly induced in PC12 cells by a number of agents (NGF, FGF, EGF, TPA, insulin, dbcAMP, Ca^{2+}-ionophores; [163,164]). Since EGF, TPA, insulin, and Ca^{2+} do not cause neurite formation, it is clear that c-*fos* induction is not sufficient for neuronal differentiation. Moreover, expression of an activated N-Ras protein stimulates neurite outgrowth of PC12 cells without activating c-*fos* transcription [66]. Microinjection of an anti-Fos antibody into PC12 cells slightly increased their ability to develop neurites upon NGF treatment, while DNA synthesis was markedly inhibited [139]. Taken together, these observations support the notion that c-Fos is likely to be involved in the proliferation rather than neuronal differentiation of PC12 cells.

The proto-oncogene c-*jun*, a member of the *jun*-family of early-response genes codes for another leucine zipper protein that acts as homodimer or forms heterodimers with *fos*-family members in AP-1 (see above). Embryonic stem cells lacking a functional c-*jun* gene are viable [165], but homozygous c-*jun*-deficient mice die in utero [166] indicating that c-Jun plays a vital role in certain tissues but not in others. c-*jun* transcription is induced by NGF, FGF, EGF, dbcAMP and TPA in PC12 cells [69,164,167], but its function in neuronal differentiation remains to be determined.

The early-response gene *zif268* (also known as *egr-1*, NGFI-A, Krox-24, TIS8, 225, d-2, GOS30; reviewed in [144]) was isolated from cDNA libraries enriched for serum growth factor-inducible sequences. It is induced by mitogenic agents in all mammalian cells studied. Many early- and secondary-response gene promoters contain Zif268-binding sites recognized by the zinc finger-containing DNA-binding domain of the protein [168]. These observations make Zif268 an excellent third messenger candidate. *zif268* is induced by NGF, FGF, EGF, and TPA in PC12 cells [69,164]. Induction by all three growth factors is Ras-dependent ([164]; J. Szeberényi, and G.M. Cooper, unpublished data), the effect of NGF is mediated by the Trk-Src-Ras-Raf pathway [49]. The nuclear form of Zif268 protein is strongly phosphorylated in NGF-stimulated cells [169]. Further studies on Zif268 expression and phosphorylation are needed to determine the significance of these events in NGF-signaling.

nur77 (NGFI-B, N10, TIS1, reviewed in [144]), another early-response gene identified by screening of cDNA libraries, codes for an orphan receptor of the thyroid/steroid receptor family; its ligand is yet to be identified. Its complex promoter [170,171] can be activated by a variety of agents in PC12 cells (NGF, EGF, FGF, TPA, depolarization; [164,172]). Phosphorylation and subsequent nuclear translocation of Nur77 proteins are induced only by growth factors with neuritogenic potential (NGF, FGF), but not by other agents (EGF, TPA, Ca^{2+}-ionophore; [172]), suggesting a role for this protein in differentiation. NGF stimulates both the expression and phosphorylation of Nur77 via Ras-independent pathways (J. Szeberényi, and G.M. Cooper, unpublished data).

2.5.4. Induction of secondary-response genes

Besides early-response genes, growth factor stimulation of target cells leads to the activation of a second set of genes, late- or secondary-response genes (see above). This battery of genes differs from early-response genes in several aspects. First, their induction is delayed: it usually starts after hours and peaks after days of growth factor treatment. Second, transcriptional activation of these genes is absolutely dependent on protein synthesis indicating the involvement of newly synthesized transcription factor(s). Third, products of these genes are directly implicated in the appearance of growth factor-specific phenotypic features. This point is indirectly supported by the observation that, while early-response genes are activated by a wide variety of agents, secondary-response genes are less promiscuous, their inducibility being restricted to a few stimuli.

A number of secondary-response genes are induced in NGF-treated PC12 cells (for review see [159]). They include genes coding for neurofilament proteins NF-M and NF-L [173], the neuronal-specific intermediate filament protein peripherin [174], the membrane-bound growth cone protein SCG10 [175,176], the small, ubiquitous peptide thymosin [177] and Ca^{2+}-binding proteins [178].

The gene coding for *transin* is the prototypical secondary-response gene. Transin (stromelysin) belongs to the family of secreted matrix metalloproteinases, a group of enzymes whose dysfunction is implicated in degenerative diseases of connective tissues (e.g., arthritis) and in the dissemination of invasive malignant tumors (reviewed in [179]). For this reason, the regulation of transin expression is in the focus of intensive investigations. The promoter region of transin gene contains recognition sites for AP-1 and Zif268 [128], as well as enhancer elements responsive to Ras and PC-PLC [180]. In PC12 cells transin expression is tightly regulated: basal expression is low and is strongly inducible by NGF and FGF, growth factors that cause neurite outgrowth [181]. Induction of transin by NGF is independent of PK A [131] and PK C [180], but it requires intact Ras function [69]. NGF activation of transin gene expression is apparently mediated by the same Trk-Src-Ras-Raf pathway as morphological differentiation [49].

As indicators of phenotype-related events in PC12 cells, secondary-response genes are useful targets for studies in NGF signaling. Two important points, however, should be emphasized. First, while induction of transin by NGF is Src-Ras-Raf-mediated, activation of SCG10 is Raf-independent, and induction of Thy 1 is Ras- and Raf-independent [49]. Second, TPA, an agent lacking the potential of inducing neurite outgrowth, strongly activates transin gene transcription (J. Szeberényi, unpublished data). These observations suggest that secondary-response genes are differentially regulated, and that their expression may not always correlate with the phenotypic response.

Table 1
The effect of the dominant inhibitory mutant Ha-Ras Asn17 protein on NGF-induced responses in PC12 cells

NGF responses	'Low expressor' M17-subclones	'High expressor' M17-subclones
Early-response genes		
c-fos: induction of mRNA [a]	+ + +	±
induction of protein [b]	+ + +	±
phosphorylation of protein [b]	+ + +	±
c-jun: induction of mRNA [a]	+ +	±
induction of protein	n.d.	n.d.
phosphorylation of protein	n.d.	n.d.
zif268: induction of mRNA [a]	+ + +	±
induction of protein [b]	+ + +	±
phosphorylation of protein [b]	+ + +	±
nur77: induction of mRNA [b]	+ + +	+ + +
induction of protein [b]	+ + +	+ + +
phosphorylation of protein [b]	+ + +	+ + +
Secondary-response genes		
transin: induction of mRNA [a]	−	−
SCG10: induction of mRNA [a]	−	−
Neurite outgrowth [a]	±	±

[a] See [89].
[b] J. Szeberényi and G.M. Cooper, unpublished data.
+ + +, normal response (comparable to that of control PC12 cells); + +, slightly reduced response; ±, strongly reduced response; −, no response; n.d., not determined.

3. Multiple signaling pathways: with or without Ras

Biochemical analysis of PC12 cells clearly indicates, as described in the previous Section, that functional activities of a large number of proteins with signal transducing potential are modulated upon treatment of these cells with NGF. For most of these proteins, however, it is presently not known whether they really have a significant contribution to NGF signaling, and only a couple of proteins have been found to be absolutely required for NGF-induced neuronal differentiation (see Fig. 1). In addition, an increasing body of evidence supports the notion, that, instead of being arranged in a linear order, signaling proteins are aligned along multiple parallel, but interlocking pathways. Such a complex network of signaling wires makes it extremely difficult to tell vital major pathways from unimportant accessory routes, and to determine the connections between signaling molecules. One possible way to overcome such difficulties is to selectively block individual pathways and to study the consequences.

Ras proteins are among the few whose function has unequivocally been proved to be indispensable for NGF-stimulated neuronal differentiation of PC12 cells (see Section 2.3). Expression of detectable levels of the dominant inhibitory Ha-Ras Asn-17 protein in PC12 cells completely blocks NGF-induced neurite formation [69]. Somewhat unexpectedly, the mutant protein was found to have no effect on the proliferation of PC12 cells ([69]; in sharp contrast Ha-Ras Asn-17 strongly inhibits the growth of NIH 3T3 fibroblasts [73]). The lack of growth-inhibition made it possible to isolate stable PC12 transfectants expressing different levels of the mutant protein (designated M17-subclones) and to study the Ras-dependence of NGF-stimulated biochemical changes accompanying neuronal differentiation [69,89]. M17-subclones can be, somewhat arbitrarily, classified in two main categories (Table 1): in 'low expressor' subclones the relatively low level of Ha-Ras Asn-17 is sufficient to block NGF induction of the secondary-response gene transin and neurite outgrowth, but induction of the prototypi-

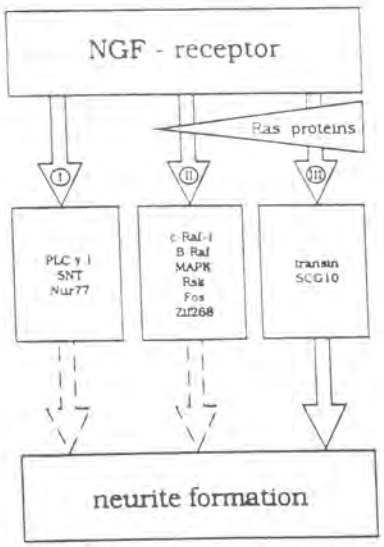

Fig. 2. Ras-dependent and -independent pathways in NGF signaling. The triangle representing Ras proteins indicates that pathways I, II and III are Ras-independent or require small and large amounts of activated Ras, respectively. Pathway III is essential for NGF-induced neurite formation (indicated by the solid-line arrow), while the requirement for pathways I and II in neuritogenesis is not clear (broken-line arrows). Abbreviations: see footnote.

cal early-response gene c-*fos* (and several other genes of this type) by NGF proceeds normally. In contrast, all these responses are severely impaired in M17-subclones expressing high levels of the mutant protein designated ('high expressor' subclones, see Table 1). Such PC12 cell lines expressing different levels of the interfering mutant Ras protein provide a useful model system to dissect mechanisms of signaling from the NGF receptor by selectively inhibiting pathways involved in the signal transduction process.

Comparison of NGF-responses of control PC12 cells, 'low' and 'high expressor' M17-subclones [89], made it possible to tentatively identify and partially characterize three main signal transduction pathways that originate from the NGF receptor (Fig. 2).

Pathway I is independent of Ras function. This signaling route was identified by treating 'high expressor' M17-subclones with a combination of NGF and dbcAMP, or NGF and ionomycin (a calcium ionophore). While NGF is unable to induce neurite formation in these cells, it strongly synergizes with both second messenger analogs for morphological differentiation [89]. NGF activation of this pathway by itself is not sufficient to induce neuronal differentiation, since even low levels of Ha-Ras Asn-17 render PC12 cells unresponsive to NGF-stimulated neurite outgrowth. Stimulation of pathway I is also insufficient to fully activate the early-response genes c-*fos*, c-*jun* and *zif268*: induction of these genes is almost completely blocked in high Ha-Ras Asn-17 expressor cell lines (Table 1). This pathway works well in PK C downregulated cells, and it also appears distinct from cAMP- or calcium-dependent signaling mechanisms, since it strongly synergizes with both second messengers [89]. The role of Ras-independent biochemical changes in NGF stimulated cells (e.g., tyrosine-phosphorylation and activation of PLCγ [88,89], tyrosine-phosphorylation of SNT [41], induction and phosphorylation of Nur77 (J. Szeberényi and G.M. Cooper, unpublished data) in mediating this pathway is yet to be determined. Neither has the significance of pathway I in NGF-induced neuronal differentiation been determined yet.

Pathway II, a weakly Ras-dependent route, was identified by comparing NGF-induced c-*fos* expression in PC12 cells expressing low or high concentrations of Ha-Ras Asn-17 protein: this pathway is required for full induction of early-response genes by NGF and is inhibited in high Ha-Ras Asn-17 expressors only. PK C downregulation experiments using a low expressor M17-subline, in which this signaling route is functional, suggest that this pathway does not require the function of PK C [89]. Pathway II is sufficient to mediate normal expression and phosphorylation of c-Fos and Zif268 proteins (J. Szeberényi and G.M. Cooper, unpublished data). Its contribution to morphological differentiation is presently not clear.

Pathway III represents a strongly Ras-dependent signaling mechanism, since it is blocked in all M17-subclones with detectable levels of Ha-Ras Asn-17 expression. It is required for NGF induction of morphological differentiation and secondary-response genes by NGF (see Table 1). Pathway III is not PK C-dependent, since direct stimulation of PK C by TPA, in combination with NGF, does not bypass the block of neuronal differentiation in PC12 cells expressing low amounts of Ha-Ras Asn-17 [89]. Neither is it mediated by cAMP-dependent PK A, since a PC12 subclone deficient in PK A function still responds to NGF treatment with morphological differentiation [182].

The signaling components mediating the two putative Ras-dependent pathways (II and III, Fig. 2) are presently unknown. Ras-control of the Raf-MAPK-Rsk cascade is strongly supported by experimental evidence (see Section 2), but the role of this cascade in NGF-induced early-response gene activation on one hand, and secondary-response gene induction and neurite formation on the other one, is not clear. One possible scenario is that pathways II and III diverge right after Ras and are mediated by different signal transducing molecules. It is also concievable, however, that divergence of the pathways happens further down the line, and both routes are mediated by the Raf-MAPK-Rsk cascade. If this was the case, differential inhibition of the two pathways by Ha-Ras Asn-17 may be caused by their having different threshold values for activation: partial inhibition of the protein kinase cascade would

then be sufficient to block neuritogenesis, while inhibition of early-response gene induction would require a more pronounced reduction of phosphorylation events along the pathway. Detailed analysis of these protein kinases in a series of M17-subclones is expected to differentiate between these two possiblities.

4. Future perspectives

Things are cleary on the move now in NGF-signaling: several major discoveries (identification of $p140^{trk}$ as NGFR; analysis of upstream connections of Ras proteins; identification of Ras-Raf-MAPK-Rsk as a major signaling cascade in PC12 cells) during the last 2–3 years started to clarify events on the proximal end of the signaling network. Here are a few fields where further breakthroughs can be expected in the near future.

(I) The nature of the functional, high affinity NGF receptor is still a matter of controversy (see Section 2.1). The debate over the Trk homodimer vs. Trk-p75 heterodimer theories may be settled by targeted disruption of the relevant genes in PC12 cells and in transgenic animals. The neurotrophin receptor-specific protein kinase inhibitor $K252_a$ will also be a useful tool to probe $p140^{trk}$ function in NGF signaling [183].

(II) As mentioned above, the real involvement of most of the NGF modulated signaling proteins in the process of neuronal differentiation has not yet been established. The exact function of these proteins in NGF signaling will be studied by using dominant negative mutations or knock-out-mutations in their genes.

(III) Such mutants will be especially useful in identifying in vivo substrates of specific protein kinases (e.g., Rafs, MAPKs, Rsk). These experiments will be absolutely critical to unveil in vivo connections between protein kinases and their targets, since most putative protein kinase substrates so far have been identified by in vitro phosphorylation reactions. The in vivo relevance of results optained by such assays should always be carefully evaluated, and ultimately confirmed by in vivo experiments.

(IV) The exact relationships between NGF induction of early- and secondary-response genes and the phenotypic response will be thoroughly analyzed. The observation that enucleated PC12 cells respond to NGF treatment with transient process formation indicates that pre-existing neurite components can initiate neurite outgrowth, but new gene expression is required to maintain morphological differentiation [184]. Details of the differential regulation and individual contribution of early- and secondary-response genes to the biological response of PC12 cells to NGF is expected to be the target of intensive studies.

(V) One of the most intriguing questions in NGF signaling is this: what biochemical mechanisms provide the specificity required for the development of the phenotypic response of PC12 cells to NGF treatment: neuronal differentiation? NGF, a differentiating agent, and EGF, a mitogenic growth factor, trigger similar, sometimes indistinguishable biochemical events in PC12 cells (e.g., increase in Ras-GTP, stimulation of Raf and MAPK activities, expression of a similar set of early-response genes, etc.) that ultimately lead to completely different, growth factor specific biological responses. The exact biochemical basis for the specificity of NGF action will be determined by looking for subtle differences between the surprisingly similar mitogenic signal transduction of EGF and neuritogenic signaling events in NGF-treated PC12 cells [185].

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Differential Regulation of Raf-1 and B-Raf and Ras-Dependent Activation of Mitogen-Activated Protein Kinase by Cyclic AMP in PC12 Cells

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Growth factor stimulation of the mitogen-activated protein (MAP) kinase pathway in fibroblasts is inhibited by cyclic AMP (cAMP) as a result of inhibition of Raf-1. In contrast, cAMP inhibits neither nerve growth factor-induced MAP kinase activation nor differentiation in PC12 pheochromocytoma cells. Instead, in PC12 cells cAMP activates MAP kinase. Since one of the major differences between the Ras/Raf/MAP kinase cascades of these cell types is the expression of B-Raf in PC12 cells, we compared the effects of cAMP on Raf-1 and B-Raf. In PC12 cells maintained in serum-containing medium, B-Raf was refractory to inhibition by cAMP, whereas Raf-1 was effectively inhibited. In contrast, both B-Raf and Raf-1 were inhibited by cAMP in serum-starved PC12 cells. The effect of cAMP is thus dependent upon growth conditions, with B-Raf being resistant to cAMP inhibition in the presence of serum. These results were extended by studies of Rat-1 fibroblasts into which B-Raf had been introduced by transfection. As in PC12 cells, B-Raf was resistant to inhibition by cAMP in the presence of serum, whereas Raf-1 was effectively inhibited. In addition, the expression of B-Raf rendered Rat-1 cells resistant to the inhibitory effects of cAMP on both growth factor-induced activation of MAP kinase and mitogenesis. These results indicate that Raf-1 and B-Raf are differentially sensitive to inhibition by cAMP and that B-Raf expression can contribute to cell type-specific differences in the regulation of the MAP kinase pathway. In contrast to the situation in PC12 cells, cAMP by itself did not stimulate MAP kinase in B-Raf-expressing Rat-1 cells. The activation of MAP kinase by cAMP in PC12 cells was inhibited by the expression of a dominant negative Ras mutant, indicating that cAMP acts on a target upstream of Ras. Thus, it appears that a signaling component upstream of Ras is also required for cAMP stimulation of MAP kinase in PC12 cells.

Signal transduction by receptor protein-tyrosine kinases is linked to activation of the Raf/MEK/mitogen-activated protein (MAP) kinase cascade by the Ras guanine nucleotide-binding proteins (18, 22). Growth factor binding results in receptor autophosphorylation, leading to the association of adaptor protein-guanine nucleotide exchange factor complexes with the activated receptors. Then the guanine nucleotide exchange factors catalyze the formation of the active Ras-GTP complex from inactive GDP-bound Ras. GTP-bound Ras interacts directly with Raf (23, 41–43, 45), thereby recruiting Raf to the plasma membrane where it is activated by currently unidentified signals (6, 21, 36). Then Raf phosphorylates and activates MEK (9, 17, 20), which in turn phosphorylates and activates the p44 and p42 MAP kinases Erk1 and Erk2, respectively. Then the MAP kinases phosphorylate and regulate a diverse group of proteins, including other protein kinases (such as pp90 S6 kinase), phospholipases (such as PLA$_2$), and transcription factors (such as Elk-1) (3).

The prototype member of the Raf family, Raf-1, is ubiquitously expressed and appears to be a MEK activator common to a variety of different cell types (37). However, several other MEK-activating kinases are expressed in a cell- or tissue-specific manner. These include Mos (26, 29, 34), which is specifically expressed in germ cells (11, 25, 30), as well as two additional members of the Raf family, A-Raf and B-Raf (2, 35), which are expressed principally in urogenital tissues and in neuronal tissues and testes, respectively (37). The tissue-specific expression of these MEK-activating kinases suggests that they play a role in the differential regulation of MAP kinase in different cell types.

Previous studies have demonstrated cross talk between the Ras/Raf/MEK/MAP kinase cascade and the cyclic AMP (cAMP) second messenger system at the level of Raf-1. This has been delineated in several cell types, including Rat-1 and NIH 3T3 fibroblasts, rat adipocytes, and human arterial smooth muscle cells (5, 7, 13, 33, 44). In these cells, cAMP inhibits the activation of Raf-1, resulting in the inhibition of both growth factor-stimulated cell proliferation and MAP kinase activation. It has further been shown that Raf-1 is phosphorylated by cAMP-dependent protein kinase (PKA) at a consensus site within the Raf-1 regulatory domain (44). This phosphorylation reduces the affinity with which Raf-1 binds to Ras, suggesting that it is responsible for cAMP-mediated inhibition of Raf-1 (44).

While growth factors and cAMP have antagonistic effects on fibroblasts, both nerve growth factor (NGF) and cAMP can induce neuronal differentiation of PC12 pheochromocytoma cells (14, 15, 31). Moreover, combined treatment with NGF plus cAMP has a synergistic effect on neurite formation (15, 31). The induction of differentiation by NGF is mediated by the Ras/Raf/MEK/MAP kinase cascade, because dominant inhibitory mutants of Ras and MEK interfere with NGF-induced differentiation (8, 38) whereas constitutively active mutants of Ras, Raf, and MEK are themselves sufficient to induce differentiation (1, 8, 27, 39). In contrast to NGF, the mechanism by

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which cAMP induces differentiation is unclear; previous reports have disagreed as to whether cAMP stimulates MAP kinase activity in PC12 cells (10, 40). However, it appears that in contrast to the situation in fibroblasts, the stimulation of MAP kinase by NGF in PC12 cells is not inhibited by cAMP (10, 40).

One of the major differences between the Ras/Raf/MEK/MAP kinase cascades in PC12 cells and Rat-1 fibroblasts is the presence of B-Raf in PC12 cells, in which it appears to be the major MEK activator (19, 24). The PKA consensus site found in the regulatory domain of Raf-1 is not present in B-Raf (35), so B-Raf might be expected to be resistant to inhibition by cAMP. We therefore initiated experiments to compare the effects of cAMP on Raf-1 and B-Raf.

During the course of these studies, it was reported that B-Raf as well as Raf-1 is inhibited by the treatment of PC12 cells with cAMP agonists (28, 40). Those experiments were carried out with cells maintained in serum-deficient medium lacking growth factors for 16 to 20 h. We report here that the effects of cAMP on B-Raf are dependent upon growth conditions for both PC12 cells and transfected B-Raf-expressing Rat-1 fibroblasts. While Raf-1 is effectively inhibited by cAMP, B-Raf is resistant to inhibition by cAMP under normal growth conditions. Thus, it appears that Raf-1 and B-Raf are differentially regulated by cAMP and that the relative insensitivity of B-Raf to cAMP contributes to cAMP stimulation of the MAP kinase pathway in PC12 cells. However, the expression of B-Raf in Rat-1 cells was not sufficient to make MAP kinase inducible by cAMP alone, whereas cAMP itself was able to stimulate MAP kinase in PC12 cells. This stimulation of MAP kinase by cAMP in PC12 cells was Ras dependent, indicating that cell-specific factors acting upstream of Ras are also required for cAMP stimulation of the MAP kinase pathway.

MATERIALS AND METHODS

Cell lines and transfections. PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% heat-inactivated fetal bovine serum. Rat-1 cells were transfected by calcium phosphate precipitation with 10 μg of a Moloney murine leukemia virus-based vector expressing normal human B-Raf cDNA (LTR-neo B-Raf). G418-resistant colonies were isolated 4 weeks after transfection and analyzed for B-Raf expression. Both normal and transfected Rat-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. When starved, cells were incubated in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin for 16 to 20 h (40).

In-gel MAP kinase assay. The in-gel MAP kinase assay was carried out as described previously (12) with minor modifications. Briefly, 10 or 20 μg of sonicated cell extracts was electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel containing 0.5 mg of myelin basic protein per ml copolymerized into the separating gel. After electrophoresis, the gel was washed twice each in 20% isopropanol in 50 mM Tris-HCl (pH 8) in buffer A (50 mM Tris-HCl [pH 8], 5 mM β-mercaptoethanol) and then in 6 M guanidine hydrochloride in buffer A. The proteins were allowed to renature at 4°C by extensive washing in buffer A containing 0.04% Tween 40. After overnight renaturation, the myelin basic protein kinase activity was measured by incubating the gel in kinase buffer (40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 2 mM dithiothreitol, 15 mM MgCl$_2$, 100 μM EGTA) containing 10 μCi of [γ-^{32}P]ATP. Gels were washed extensively in 5% trichloroacetic acid–1% sodium PP$_i$, and the incorporated radioactivity was quantitated by using a PhosphorImager.

Raf-1 and B-Raf kinase assays. Raf-1 was immunoprecipitated from extracts of 10^5 to 10^6 PC12 cells or 10^5 Rat-1 cells with 4 μl of anti-Raf-1 monoclonal antibody (Transduction Laboratories) as described previously (6), and antigen-antibody complexes were collected on protein A-Sepharose beads. Immunoprecipitates were resuspended in 40 μl of 0.5 mM β-glycerophosphate (pH 7.3)–1.5 mM EGTA–1 mM dithiothreitol–0.03% Brij 35. Then the phosphorylation of MEK was assayed in reaction mixtures containing 40 μl of Raf-1-immunoprecipitated beads, 10 μl of 50 mM MgCl$_2$, 2 μl (10 Ci/mmol) and 0.3 μg of wild-type MEK (Santa Cruz Biotechnology). Reaction mixtures were incubated at 30°C for 30 min, beads were removed by centrifugation, and supernatant fluids were electrophoresed on SDS–10% polyacrylamide gels. The phosphorylation of MEK was quantitated by using a PhosphorImager.

B-Raf kinase assays followed the same protocol except that the antibody used was anti-B-Raf polyclonal antibody and only 20 μl of immunoprecipitates was added to kinase reaction mixtures.

Immunoblot analysis. Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 7.5]) supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 μg of aprotinin per ml. Protein samples (50 μg) were electrophoresed on SDS–10% polyacrylamide gels, and proteins were transferred to nitrocellulose filters. Then filters were blocked with phosphate-buffered saline containing 3% skim milk and 0.2% Tween 20 and probed with anti-B-Raf polyclonal antibodies or anti-Raf-1 monoclonal antibodies. Filters were washed, and antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G.

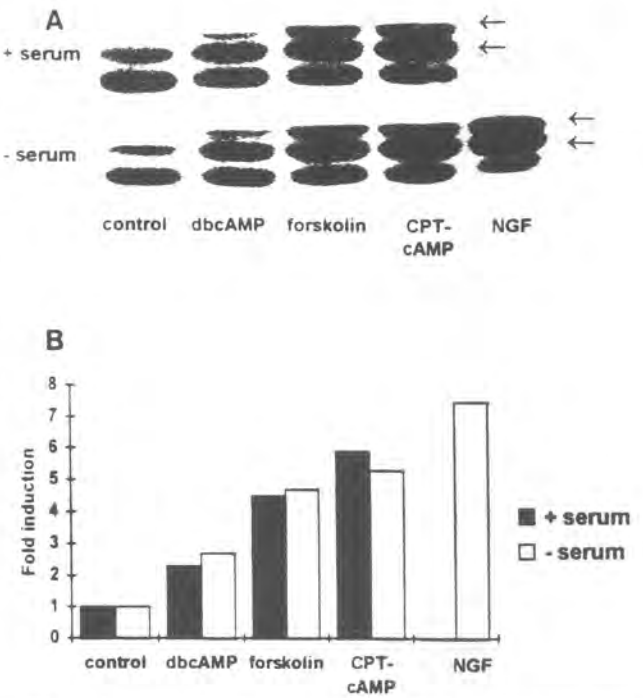

FIG. 1. Activation of MAP kinase by cAMP in PC12 cells. Cells were maintained in normal growth medium (+ serum) or incubated in serum-free medium for 20 h (− serum). Cells were incubated with dbcAMP (0.5 mM), forskolin (50 μM), CPT-cAMP (0.5 mM), or NGF (100 ng/ml) for 5 min or left untreated (control). The MAP kinase activities in cell lysates were determined by in-gel kinase assays (A) and quantitated by using a PhosphorImager (B). The results are presented as the fold induction of MAP kinase compared with that of untreated controls. The positions of the p42 and p44 MAP kinases are indicated by arrows.

RESULTS

Effect of cAMP agonists on MAP kinase activation in PC12 cells. Since previous reports (10, 40) disagreed as to whether cAMP alone could activate MAP kinase in PC12 cells, we first determined the effect of cAMP treatment on MAP kinase activity. Cells were treated for 5 min with cAMP agonists with different mechanisms of action and membrane permeabilities to exclude possible nonspecific effects of the treatment. Dibutyryl-cAMP (dbcAMP) and chlorophenylthio-cAMP (CPT-cAMP) are membrane-permeable cAMP derivatives which act to increase intracellular cAMP levels, while forskolin is a direct activator of the catalytic subunit of PKA. Then the activation of MAP kinase was measured by an in-gel kinase assay in which retarded migration of the phosphorylated enzyme is evident as retarded bands of phosphorylated myelin basic protein (12).

All three cAMP agonists used in these experiments stimulated MAP kinase activity (Fig. 1). The most effective was the

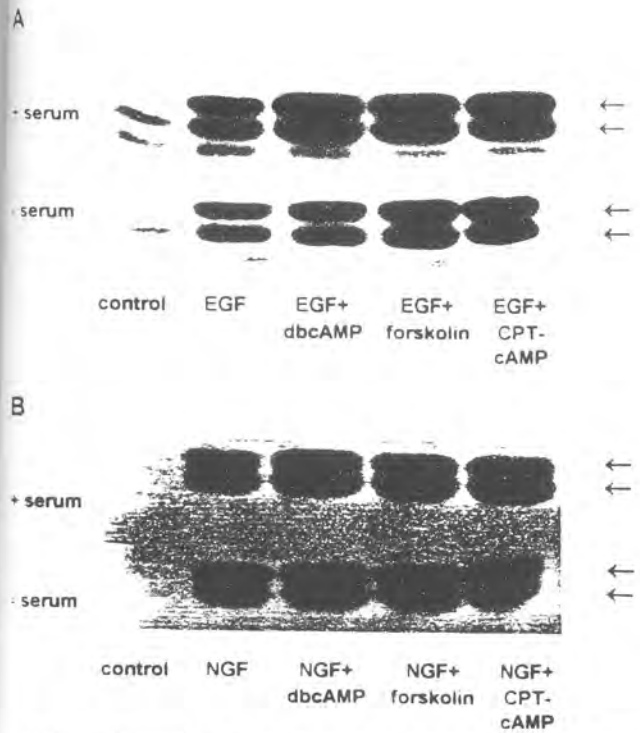

FIG. 2. Effect of cAMP on growth factor-stimulated MAP kinase activity in PC12 cells. Cells were maintained under normal growth conditions (+ serum) or in serum-free medium (- serum) and treated with cAMP agonists as described in the legend to Fig. 1 for 10 min. EGF (50 ng/ml) (A) or NGF (100 ng/ml) (B) was then added for 5 min. Controls were left untreated. MAP kinase activities were determined as described in the legend to Fig. 1. The positions of the p42 and p44 MAP kinases are indicated by arrows.

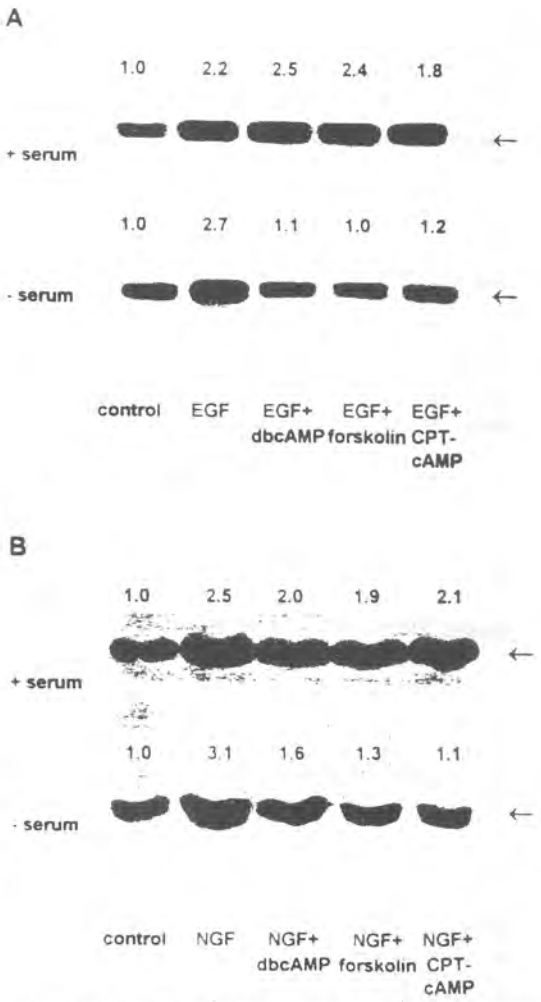

FIG. 4. Effect of cAMP on growth factor-induced B-Raf kinase activity in PC12 cells. Cells were maintained under normal growth conditions (+ serum) or in serum-free medium (- serum) and treated as described in the legend to Fig. 2. B-Raf kinase activities were determined by immunokinase assays. The results were quantitated by using a PhosphorImager and are presented above the corresponding gel as the fold induction of B-Raf kinase compared with that of untreated controls. The positions of phosphorylated MEK bands are indicated by arrows.

most membrane-permeable derivative, CPT-cAMP, which was nearly as potent a MAP kinase activator as NGF. Forskolin was a slightly weaker MAP kinase activator than CPT-cAMP was, while the activity of dbcAMP was about half that of the other two cAMP agonists (Fig. 1). Similar extents of MAP kinase activation were observed in cells cultured in serum-free medium for 20 h as described by Vaillancourt et al. (40) and in cells maintained in normal growth medium (containing 10% horse serum and 5% fetal bovine serum).

We then determined the effect of cAMP in combination with either epidermal growth factor (EGF) or NGF on MAP kinase activity, again in both normal growth medium and serum-free medium. Cells were first pretreated with cAMP agonists for 10 min, and then EGF or NGF was added to the culture medium for an additional 5 min. As reported previously (10, 40), cAMP treatment did not inhibit MAP kinase activation by either the mitogenic EGF (Fig. 2A) or the neurotrophic NGF (Fig. 2B). Instead, cAMP appeared to synergize with the polypeptide growth factors to further increase MAP kinase activity.

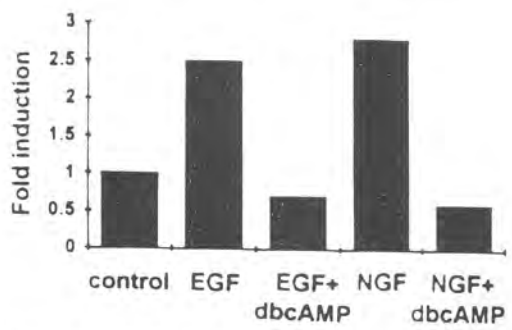

FIG. 3. Effect of cAMP on growth factor-induced Raf-1 kinase activity in PC12 cells. Cells maintained under normal growth conditions (serum-containing medium) were treated with 0.5 mM dbcAMP or left untreated for 10 min. EGF (50 ng/ml) or NGF (100 ng/ml) was then added for an additional 5 min. Raf-1 kinase activities were determined by immunokinase assays. The results were quantitated by using a PhosphorImager and are presented as the fold induction of Raf-1 kinase compared with that of untreated controls.

Differential regulation of Raf-1 and B-Raf by cAMP in PC12 cells. To investigate the effect of cAMP on the activation of Raf isotypes under different growth conditions, we compared the in vitro MEK kinase activities of Raf-1 and B-Raf immunoprecipitates of PC12 cells cultured in serum-rich medium and serum-free medium. Cells were treated with EGF or NGF for 5 min and, as indicated, pretreated with cAMP agonists for 10 min. The activation of Raf-1 in NGF- and EGF-treated cells was strongly inhibited by cAMP agonists in both normal growth medium (Fig. 3) and serum-free medium (data not

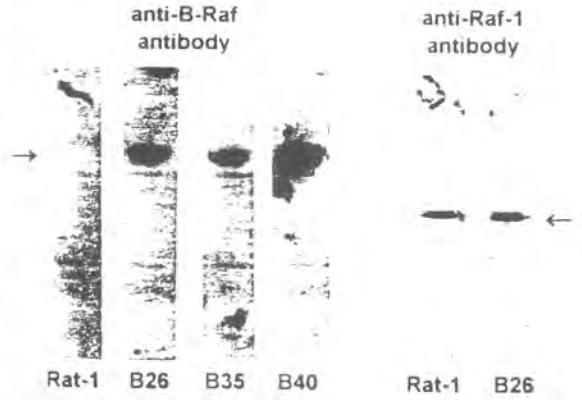

FIG. 5. Expression of B-Raf and Raf-1 in Rat-1 cell lines. The expression of B-Raf and Raf-1 was analyzed by immunoblotting extracts of normal Rat-1 cells and of three Rat-1 cell clones (B26, B35, and B40) that had been transfected with B-Raf cDNA. Raf-1 and B-Raf bands are indicated by arrows on the right and left, respectively.

shown). In both cases, Raf-1 kinase activity was reduced below the basal level in cells treated with dbcAMP.

In contrast, B-Raf was refractory to inhibition by cAMP agonists when cells were maintained in serum-containing medium (Fig. 4). However, as previously reported (28, 40), cAMP did inhibit B-Raf activity in serum-starved cells (Fig. 4). The effect of cAMP on B-Raf is thus dependent upon the growth conditions of cells. Under normal conditions in the presence of serum, B-Raf is refractory to inhibition by cAMP, whereas Raf-1 is effectively inhibited.

Differential regulation of Raf-1 and B-Raf in Rat-1 cells expressing B-Raf. The results discussed above indicate a differential regulation of Raf isotypes in PC12 cells, with B-Raf being more resistant to inhibition by cAMP than Raf-1 is. To extend these observations, we investigated the effect of cAMP on Raf-1 and B-Raf in Rat-1 fibroblasts, in which cAMP inhibits MAP kinase activation (5, 7, 44). Normal Rat-1 cells expressed Raf-1 but not B-Raf (Fig. 5). To obtain B-Raf-expressing cells, normal Rat-1 cells were transfected with an expression plasmid containing full-length human B-Raf cDNA. Then G418-resistant transfected colonies were isolated and tested for B-Raf expression by immunoblot analysis (Fig. 5). Three transfected clones (designated B26, B35, and B40) expressing significant levels of B-Raf, which at least reached the level of B-Raf in PC12 cells, were chosen for further analysis.

The effects of cAMP agonists on Raf-1 and B-Raf kinase activities in transfected Rat-1 cells were then tested both in normal growth medium (supplemented with 10% calf serum) and after 20 h of incubation in the absence of serum. In normal Rat-1 cells, no B-Raf kinase activity was detected (data not shown). As previously reported (5, 7, 44), the stimulation of Raf-1 by EGF was completely blocked by treatment with cAMP agonists both under normal growth conditions and after serum starvation (Fig. 6A). The activation of Raf-1 was similarly inhibited by cAMP in B-Raf-transfected Rat-1 cells (data not shown). In contrast, B-Raf was resistant to inhibition by cAMP agonists in transfected Rat-1 cells maintained under normal growth conditions (Fig. 6B). However, as in the case of PC12 cells, cAMP inhibited B-Raf activation in serum-starved Rat-1 cells (Fig. 6B). Thus, as in PC12 cells, the response of B-Raf to cAMP in Rat-1 cells is dependent upon growth conditions, with B-Raf being refractory to inhibition by cAMP in cells maintained in the presence of serum.

Effects of cAMP on MAP kinase activation and mitogenesis in Rat-1 cells expressing B-Raf. In PC12 cells, cAMP potentiates both the activation of MAP kinase and the biological response to growth factors, differentiation in this case. In contrast, cAMP inhibits both MAP kinase activation and growth factor-stimulated proliferation of Rat-1 cells (5, 7, 44). Since the presence of B-Raf in PC12 cells is one major difference in the MAP kinase pathways of these two cell types, we investigated the effects of cAMP on MAP kinase activation and growth factor-induced mitogenesis in Rat-1 cells expressing B-Raf.

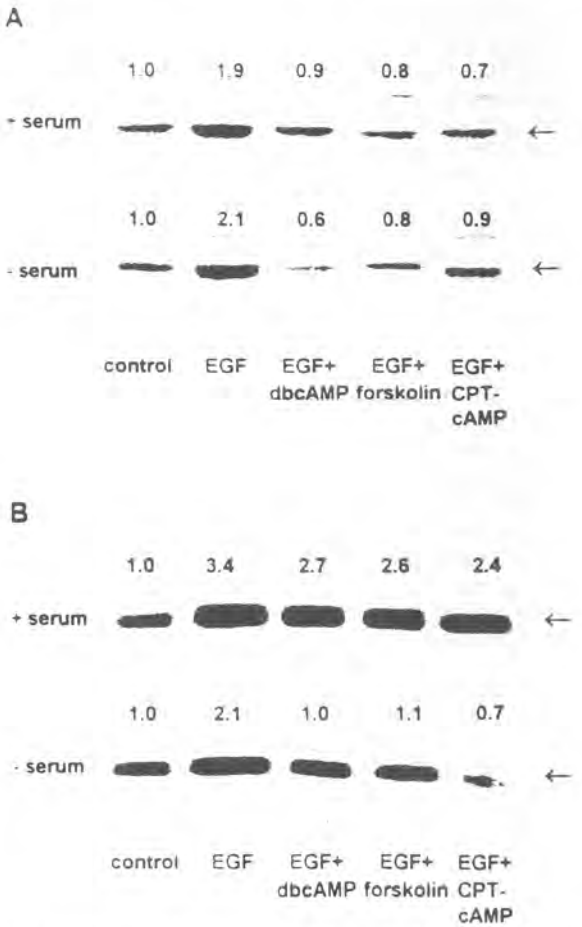

FIG. 6. Effects of cAMP on growth factor-stimulated Raf-1 and B-Raf kinase activities in Rat-1 cells. Raf-1 kinase activities were assayed in normal Rat-1 cells (A), and B-Raf kinase activities were assayed in B26 cells (B). Cells were maintained under normal growth conditions (+ serum) or in serum-free medium (- serum) and treated as described in the legend to Fig. 2. Both Raf-1 and B-Raf kinase activities were determined by immunokinase assays. The results were quantitated by using a PhosphorImager and are presented above the corresponding autoradiogram as the fold induction compared with that of the control. The positions of phosphorylated MEK bands are indicated by arrows.

In normal Rat-1 cells, the activation of MAP kinase by EGF was inhibited by cAMP agonists both under normal growth conditions and after serum starvation (Fig. 7A). In contrast, the stimulation of MAP kinase was resistant to inhibition by cAMP agonists in B-Raf-expressing Rat-1 cells (Fig. 7B). Interestingly, the activation of MAP kinase was not only resistant to inhibition by cAMP under normal growth conditions but also at least partially resistant in serum-starved cells.

To confirm these observations, we repeated similar experiments with two additional clones of Rat-1 cells expressing B-Raf. Similar results were obtained with all three indepen-

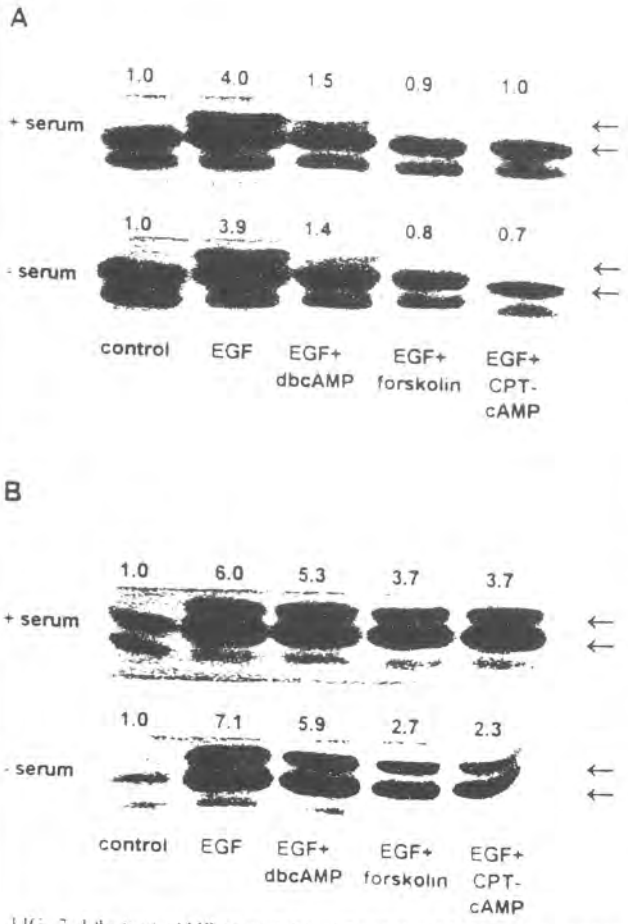

FIG. 7. Effects of cAMP on growth factor-stimulated MAP kinase activities in Rat-1 cells. Normal Rat-1 cells (A) and B26 cells (B) were maintained under normal growth conditions (+ serum) or in serum-free medium (− serum). Cells were treated as described in the legend to Fig. 2, and MAP kinase activities were determined by in-gel kinase assays. The results were quantitated by using a PhosphorImager and are presented above the corresponding autoradiogram as the fold induction compared with that of the control. The positions of the p42 and p44 MAP kinases are indicated by arrows.

dent B-Raf-expressing Rat-1 clones (Fig. 8). In the presence of serum, MAP kinase activation in the presence of cAMP was 40% of that obtained in the absence of cAMP (average of four experiments with three cell clones [standard deviation, ±8.5%]). In the absence of serum, the addition of cAMP reduced the extent of MAP kinase activation to 26% ± 7% of that observed in the absence of cAMP. The expression of B-Raf in Rat-1 cells was thus sufficient to prevent cAMP from blocking growth factor stimulation of MAP kinase.

Consistent with its effects on MAP kinase, the expression of B-Raf also rendered Rat-1 cells resistant to the inhibitory effects of cAMP on mitogenesis (Fig. 9). The proliferation of normal Rat-1 cells in response to EGF was completely blocked by CPT-cAMP. In contrast, CPT-cAMP inhibited the proliferation of two B-Raf-expressing clones (B26 and B35) only partially and had no effect on the proliferation of a third clone (B40). The expression of B-Raf thus relieves the inhibitory effects of cAMP not only on MAP kinase activation but also on the biological response of Rat-1 cells to growth factors.

Activation of MAP kinase by cAMP in PC12 cells is Ras dependent. In contrast to their effects in PC12 cells, cAMP agonists did not by themselves stimulate MAP kinase activity in B-Raf-expressing Rat-1 cells (data not shown). Thus, it appears that factors in addition to B-Raf must play a role in cAMP activation of the MAP kinase pathway in PC12 cells. To determine whether Ras function was required for cAMP stimulation of MAP kinase, we investigated the effect of expression of the dominant inhibitory mutant Ras N17 (Fig. 10). The activation of MAP kinase was effectively blocked in PC12 cells expressing Ras N17 (M-M17-26 cells), indicating that the stimulation of MAP kinase by cAMP was Ras dependent. Therefore, it appears that a cell-specific factor acting upstream of Ras is required for cAMP stimulation of MAP kinase in PC12 cells.

DISCUSSION

We have reported here that two Raf isotypes, Raf-1 and B-Raf, are differentially sensitive to inhibition by cAMP and that B-Raf expression can contribute to cell type-specific differences in the regulation of the MAP kinase signaling pathway. As reported previously (5, 7, 40, 44), Raf-1 was effectively inhibited by cAMP in both PC12 and Rat-1 cells independent of growth conditions. In contrast, B-Raf was refractory to inhibition by cAMP in PC12 cells maintained in normal growth medium, although it was inhibited by cAMP in serum-starved cells as previously reported (28, 40). These results were extended by introducing B-Raf into Rat-1 cells. In B-Raf-expressing Rat-1 cells maintained in serum-rich medium, exogenously expressed B-Raf was resistant to inhibition by cAMP, although it was sensitive to cAMP in serum-starved cells. Thus, the sensitivity of B-Raf to inhibition by cAMP differed from that of Raf-1 and displayed dependence upon growth condi-

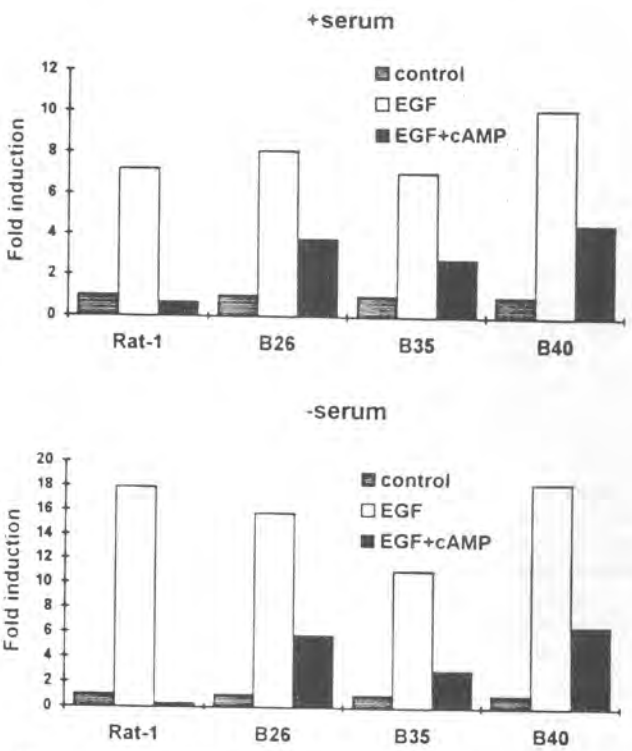

FIG. 8. Effects of cAMP on EGF-induced MAP kinase activities in independent B-Raf-expressing Rat-1 cell clones. Cells were maintained in normal growth medium (+ serum) or in serum-free medium (− serum) and treated with CPT-cAMP and EGF as described in the legend to Fig. 2. B26, B35, and B40 cells are B-Raf-expressing transfected Rat-1 cell clones. The results are presented as the fold induction of MAP kinase compared with that of untreated controls.

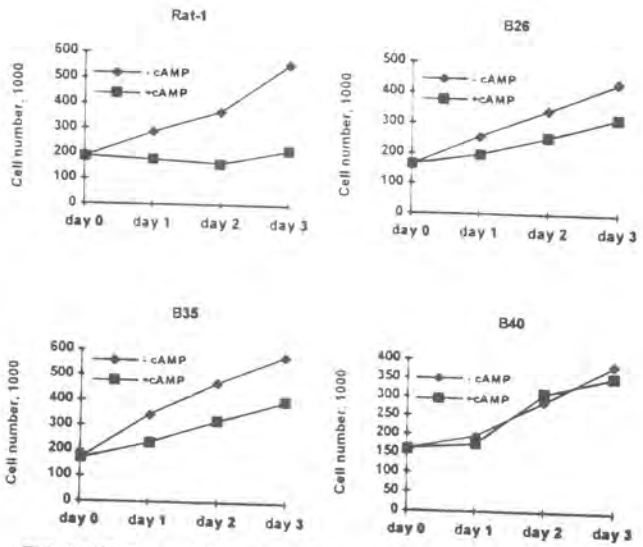

FIG. 9. Effect of cAMP on the mitogenesis of Rat-1 cells expressing B-Raf. Normal Rat-1 cells or B-Raf-expressing transfected Rat-1 clones (B26, B35, and B40 cells) were cultured in medium containing 0.5% serum for 20 h to induce quiescence. Cells were then treated with either EGF (− cAMP) or EGF plus CPT-cAMP (+ cAMP) for up to 3 days, and cell numbers were determined with a Coulter counter.

tions in both PC12 and Rat-1 cells, indicating that the relative resistance of B-Raf to cAMP inhibition is an intrinsic property of the B-Raf protein.

Raf-1 has an optimal PKA consensus site at position 43 in its N-terminal regulatory domain (4). Since this site is not present in B-Raf (35), direct phosphorylation of serine 43 by PKA may account for the specific inhibition of Raf-1 by cAMP. This is consistent with earlier data demonstrating that cAMP inhibition of Raf-1 in Rat-1 cells was accompanied by a threefold increase in phosphorylation of serine 43 (44). Moreover, phosphorylation of Raf-1 at this position reduces the apparent affinity with which it binds to Ras both in vitro and in vivo (44).

In contrast to Raf-1, B-Raf retains most of its growth factor-induced activity under normal growth conditions in cAMP-treated cells. This may be explained by the absence of the serine 43 PKA phosphorylation site in B-Raf. A different mechanism must be invoked, however, to account for the inhibition of B-Raf by cAMP in serum-starved cells. B-Raf has been found to be phosphorylated by PKA in vitro (28), and it bears a PKA phosphorylation site at serine 330, which is close to the ATP binding site in its kinase domain (35). Thus, one possibility is that C-terminal phosphorylation accounts for the inhibition of B-Raf by cAMP in serum-starved cells. Consistent with this possibility, it has been reported that PKA can phosphorylate Raf-1 at less optimal consensus phosphorylation sites within its C-terminal kinase domain, thereby inhibiting Raf-1 activity without N-terminal phosphorylation (16). Alternatively, it is possible that B-Raf is not directly affected by PKA and that cAMP treatment of serum-starved cells instead inhibits an upstream step in the pathway leading to growth factor stimulation of the Raf kinases.

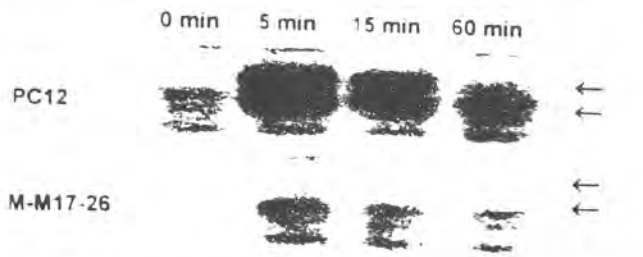

FIG. 10. Effect of a dominant inhibitory Ras mutant on MAP kinase activation by cAMP. Normal PC12 cells and PC12 cells expressing the dominant inhibitory mutant Ras N17 (M-M17-26 cells [45]) were maintained under normal growth conditions and incubated with 0.5 mM CPT-cAMP for the indicated times. MAP kinase activities were determined as described in the legend to Fig. 1. The positions of the p42 and p44 MAP kinases are indicated by arrows.

In PC12 cells, cAMP potentiates growth factor-induced neuronal differentiation and MAP kinase activation (10, 15, 31). In contrast, cAMP effectively inhibits MAP kinase activation as well as growth factor-stimulated proliferation of Rat-1 cells, which normally express only Raf-1 (5, 7, 44). The properties of the Rat-1 cells into which we introduced B-Raf by transfection indicate that the expression of B-Raf is an important determinant of the differential responses of PC12 and Rat-1 cells to cAMP. In particular, cAMP fails to effectively inhibit MAP kinase activity in B-Raf-expressing Rat-1 cells. Although some inhibition of MAP kinase was evident, its activation was never completely blocked by cAMP, even in serum-starved cells. Consistent with these results, cAMP agonists failed to effectively inhibit mitogen-induced proliferation of Rat-1 cells expressing B-Raf.

It is important to emphasize that although both Raf-1 and B-Raf are inhibited by cAMP in serum-starved cells, we did not find a corresponding inhibition of growth factor-induced MAP kinase activation. This observation is in good agreement with previous data for PC12 cells, which led to the hypothesis that PC12 cells contain an additional unidentified MEK kinase that is resistant to inhibition by cAMP (28, 40). However, our finding that the expression of B-Raf in Rat-1 cells confers cAMP resistance to MAP kinase, even after serum starvation, does not support this hypothesis. Instead, our results indicate that B-Raf expression is sufficient to confer cAMP resistance to the MAP kinase pathway without having to postulate an additional unknown MEK kinase activity. Thus, although B-Raf activity in an immunoprecipitate kinase assay was substantially inhibited by cAMP treatment of serum-starved cells, it appears that residual B-Raf activity in vivo is sufficient to result in significant stimulation of MAP kinase.

While the expression of B-Raf in Rat-1 cells was sufficient to make the MAP kinase pathway resistant to inhibition by cAMP, it did not make MAP kinase inducible by cAMP alone. Since cAMP itself can stimulate MAP kinase in PC12 cells, an additional cell-specific component of the pathway appears to be responsible for cAMP inducibility. The activation of MAP kinase by cAMP was blocked by expression of the dominant inhibitory mutant Ras N17, indicating that the cAMP-inducible component of the pathway in PC12 cells functions upstream of Ras. It is noteworthy that Ca^{2+} also stimulates the MAP kinase pathway in PC12 cells in a Ras-dependent manner (32). Further studies are required to investigate potential relationships between the cAMP and Ca^{2+} pathways and to identify the upstream signaling molecule responsible for cAMP stimulation of MAP kinase in the PC12 system.

ACKNOWLEDGMENT

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