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No Role for Ca^{++} or Protein Kinase C in *Alpha*-1A Adrenergic Receptor Activation of Mitogen-Activated Protein Kinase Pathways in Transfected PC12 Cells

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ABSTRACT

We studied the role of Ca^{++} and protein kinase C (PKC) in *alpha*-1A adrenergic receptor (AR)-mediated activation of mitogen-activated protein kinase pathways in PC12 cells. In PC12 cells stably transfected with the human *alpha*-1A AR, norepinephrine (NE) strongly activated both extracellular signal regulated kinases (ERKs) and c-jun-NH_2-terminal kinases (JNK). Ten nanomolar thapsigargin (TG) increased cytoplasmic Ca^{++} at least as much as NE but did not activate ERKs or JNK. Higher concentrations of TG caused a small activation of ERKs but not JNK. Emptying $[Ca^{++}]_i$ stores by pretreatment with TG prevented the NE-stimulated increase in $[Ca^{++}]_i$ but not ERK or JNK activation. The Ca^{++} chelator bis(2-aminophenoxy)ethane-*N-N-N′-N′*-tetraacetate (BAPTA) dose dependently abolished NE-stimulated Ca^{++} responses but not ERK or JNK activation. NE increased tyrosine phosphorylation of Pyk2, and this response was neither blocked by BAPTA nor mimicked by TG. The phorbol ester tumor promoting agent (TPA) caused a dose-dependent activation of ERKs that was potentiated by 10 nM TG. TPA caused only a small activation of JNK relative to that caused by NE, which was not affected by TG. The potent PKC inhibitor bisindolylmaleimide I dose dependently inhibited ERK and JNK activation by TPA, but not NE. ATP and UTP activated similar mitogen-activated protein kinase responses through endogenous P2Y2 receptors, and these responses were not blocked by BAPTA or bisindolylmaleimide I, suggesting that these results may be generalizable to other $G_{q/11}$-coupled receptors. The results suggest that Ca^{++} release and PKC activation are neither necessary nor sufficient for *alpha*-1A AR-mediated activation of mitogenic responses in PC12 cells.

Protein tyrosine kinases play an important role in controlling growth and differentiation (Schlessinger and Ullrich, 1992; van der Geer et al., 1994) often by activating mitogen-activated protein kinase (MAPK) signaling cascades (Seger et al., 1995). Activation of MAPK pathways by receptors with intrinsic tyrosine kinase activity is well understood, but the mechanisms by which heterotrimeric G protein-coupled receptors (GPCRs) activate these pathways are less clear.

There are several parallel MAPK pathways in cells (Robinson and Cobb, 1997). Extracellular signal regulated kinases (ERKs) 1 and 2 are activated by growth factors and cytokines and phosphorylate various molecules involved in growth and differentiation. c-Jun-NH_2-terminal kinase (JNK; also known as stress-activated protein kinase) and p38 MAPK are generally activated by stresses such as inflammatory cytokines, osmotic shock, or UV irradiation, and may inhibit cell growth and/or cause apoptosis (Xia et al., 1995). The balance between these pathways may be critical in determining cell fate.

PC12 cells have been useful in studies of growth factor-induced cellular differentiation (Wood and Roberts, 1993). Nerve growth factor (NGF) acts through tyrosine kinase receptors to cause PC12 cells to differentiate into a neuronal phenotype through a Ras-dependent activation of the ERK 1/2 cascade (Lange-Carter and Johnson, 1994). ERKs are also activated by $G_{q/11}$- and G_i-coupled receptors in PC12 cells, apparently through the tyrosine kinases Pyk2 (Lev et al., 1995) and Src (Dikic et al., 1996) in a Ras-dependent manner. We have recently shown that activation of *alpha*-1A adrenergic receptors (ARs) by norepinephrine (NE) in stably transfected PC12 cells activates ERKs, JNK, and p38 MAPK, and causes the cells to differentiate to a state indistinguishable from that caused by NGF (Williams et al., 1998). We expect these cells will be useful in studying mitogenic responses to GPCRs.

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; GPCR, G protein-coupled receptor; JNK, c-jun-NH_2-terminal kinase; NE, norepinephrine; AR, adrenergic receptor; PKC, protein kinase C; TPA, tumor promoting agent; TG, thapsigargin; BAPTA, bis(2-aminophenoxy)ethane-*N-N-N′-N′*-tetraacetate; AM, acetoxymethylester; $[Ca^{++}]_i$, intracellular calcium concentration; BSS, balanced salt solution; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; NGF, nerve growth factor; GFX 203290 or GFX, bisindolylmaleimide I; SAPK, stress-activated protein kinase.

Ca^{++} is important in growth and stress responses in many cells (Dowd, 1995; Whitaker, 1995) and may be important in MAPK responses to GPCRs (Chao et al., 1992; Rosen et al., 1994; Mitchell et al., 1995; Zohn et al., 1995; Soltoff et al., 1998). The effects of Ca^{++} in some cells are mediated by protein kinase C (PKC) (Daulhac et al., 1997; Romanelli and van de Werve, 1997; Soltoff, 1998) or calcium/calmodulin-activated protein kinase (Enslen et al., 1996), or inhibition of MAPK phosphatase-1 (Scimeca et al., 1997). Phorbol esters activate ERKs in nearly all cells, and in many cases GPCR stimulation of ERKs are at least partially blocked by inhibition or depletion of PKC (Hoshi et al., 1989; Berra et al., 1995; Hundle et al.,1995; Ueda et al., 1996; Soltoff, 1998).

Ca^{++} has been suggested to play a pivotal role in mitogenic responses in PC12 cells. Depolarization of PC12 cells has been shown to activate MAPK through a mechanism involving influx of Ca^{++} through voltage-gated channels (Rosen et al., 1994). The increased Ca^{++} activates tyrosine phosphorylation of the adapter proteins Shc and Grb2 and the epidermal growth factor receptor, promoting their association (Rosen and Greenberg, 1996). This suggests that Ca^{++} may directly activate growth factor receptor signal transduction, possibly by directly activating a tyrosine kinase such as Src. An increase in $[Ca^{++}]_i$ has also been proposed to be the key event coupling both G_i- and G_q-coupled receptor activation to MAPK activation in PC12 cells, possibly by direct activation of Pyk2 (Lev et al., 1995; Soltoff et al., 1998). Here we use *alpha*-1A AR transfected PC12 cells to clarify the role of Ca^{++} and PKC in mitogenic responses to $G_{q/11}$-coupled receptors in PC12 cells.

Materials and Methods

Materials. PC12 cells were obtained from Cindy Miranti and Michael Greenberg (Harvard Medical School). The human *alpha*-1A AR cDNA was obtained from Gozoh Tsujimoto (Tokyo). Other materials were: Lac-Switch vector system (Stratagene, La Jolla, CA); bis(2-aminophenoxy)ethane-*N*-*N*-*N*′-*N*′-tetraacetate (BAPTA) acetoxymethylester (AM) (Research Biochemicals, Natick, MA); fura-2/AM (Calbiochem, La Jolla, CA); thapsigargin (TG), (−)-NE bitartrate, EGTA, A23187, Dulbecco's modified Eagle's medium, penicillin, and streptomycin (Sigma Chemical, St. Louis, MO); phorbol-12-myristate-13-acetate (TPA) and bisindolylmaleimide I (Calbiochem, San Diego, CA); phosphospecific MAPK (Thr-202/Tyr-204) antibody, phosphospecific stress-activated protein kinase (SAPK)/JNK (Thr-183/Tyr-185) antibody, MAPK antibody, SAPK/JNK antibody (New England Biolabs, Beverly, MA); human Pyk2 antibody (Upstate Biotechnology, Lake Placid, NY); phosphotyrosine antibody (P-Y 99) and protein A-agarose conjugates, Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase (HRP)-conjugated anti-mouse IgG and enhanced chemiluminescence (ECL) reagent (Amersham, Arlington Heights, IL); HRP-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA).

Transfection. PC12 cells were cotransfected with the LacSwitch repressor (p3′SS) and the pRSVICAT operator vectors containing the human *alpha*-1A AR cDNA by lipofectamine (GIBCO/BRL, Gaithersburg, MD). Cells were propagated for several weeks in the presence of 250 μg/ml hygromycin and 500 μg/ml geneticin, and subclones expressing low constitutive and high inducible receptor levels were screened by radioligand binding.

Cell Culture. Transfected PC12 cells were propagated in 75-cm^2 flasks at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 1.4% glutamine, 20 mM HEPES, 100 mg/l streptomycin, 10^5 U/l penicillin, 10% donor horse serum, and 5% fetal bovine serum. The cells were detached by mild trypsinization (0.25%) in the presence of 2.6 mM EDTA and subcultured at a ratio of 1:3 upon reaching confluency. Except where indicated, receptor expression was induced by treatment with 1 mM isopropyl β-D-thiogalactoside at least 24 h before each experiment. For measurements of MAPK and JNK/SAPK phosphorylation, 60-mm dishes were seeded at a density of 6×10^5 cells/2 ml. For studies involving Ca^{++} measurements, 100-mm dishes were seeded at a density of 6×10^6 cells/10 ml. Cells were grown to confluency before use.

Immunoblotting. Confluent cells were serum starved for 2 h before use, and drug treatments carried out for 15 min at 37°C. After stimulation, monolayers were lysed with Laemmli sample buffer. Cell lysates were centrifuged and proteins (20 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Phosphorylation of ERKs 1 and 2 and JNK was detected by protein immunoblotting with a 1:1000 dilution of rabbit polyclonal dual phosphospecific antibodies with HRP-conjugated goat anti-rabbit IgG as a secondary antibody. Quantitation was performed by densitometry after development of membranes with ECL reagent and exposure to Hyperfilm (Amersham). NE-stimulated ERK activation in these cells has been shown to increase linearly to a maximum at 15 min and subsequently decline (Williams et al., 1998), thus 15-min time points were used in all experiments.

Pyk2 Immunoprecipitation. Confluent cells were serum starved for 2 h before further treatment. Cells were washed twice with ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate and lysed on ice with RIPA lysis buffer (1% Nonidet P-40, 25 mM HEPES, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysate was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant containing 1 mg of protein was incubated with 10 μg of anti-Pyk2 antibody at 4°C for 2 h followed by addition of 20 μl of protein A-agarose. After overnight incubation at 4°C, the sample was centrifuged and the immunoprecipitates washed three times with lysis buffer. After boiling in 30 μl of 2× SDS-sample buffer, 15 μl of supernatant was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Protein bands were detected by probing sequentially with primary antibody (anti-Pyk2, 1:1000 or anti-phosphotyrosine, 1:1000), HRP-conjugated secondary antibody (1:5000), and ECL reagent.

$[Ca^{++}]_i$ Determinations. $[Ca^{++}]_i$ transients were determined by fura-2 as described previously (Han et al., 1992; Esbenshade et al., 1993). Confluent 100 mm plates were washed with balanced salt solution (BSS; 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, 0.1% bovine serum albumin) and cells detached by mild trypsinization (0.25%). Cells were rinsed three to four times with BSS and stored on ice. One milliliter of cell suspension (1×10^6 cells/ml) was incubated with 1 μM fura-2/acetoxymethylester for 10 min at 37°C, rinsed 10 min with BSS, and diluted to 3 ml before the experiment. The cell suspension was transferred to a cuvette and placed in a Perkin-Elmer (Beaconsfield, Buckingshamshire, England) LS 50B luminescence spectrofluorometer with a thermostatted (37°C) stirred cell holder. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm. Calibration of the fluorescence signals for calculation of $[Ca^{++}]_i$ was performed by equilibrating $[Ca^{++}]_i$ and extracellular Ca^{++} with 30 μM digitonin (R_{max}), followed by addition of 30 μl of 300 mM EGTA, 1 M Tris, pH 9.0 (R_{min}), and by a K_d of 225 nM for fura-2 (Grynkiewicz et al., 1985).

Results

Effect of Ca^{++} on MAPK Phosphorylation. Treatment of PC12 cells with a Ca^{++} ionophore has been shown previously to stimulate MAPK phosphorylation (Lev et al., 1995). Fig 1 shows that in PC12 cells stably transfected with human

alpha-1A ARs, both norepinephrine (NE; 100 μM) and the Ca^{++} ionophore A23187 (10 μM) caused a large increase in ERK phosphorylation. Addition of 3 mM EGTA to chelate extracellular Ca^{++} completely abolished the effect of A23187 as expected. However, EGTA caused only a modest reduction (39%) in the effect of NE (Fig. 1), suggesting extracellular Ca^{++} does not play a major role in this response.

Effect of TG on $[Ca^{++}]_i$ and MAPK Activation. Fura-2 was used to monitor $[Ca^{++}]_i$ in suspensions of *alpha*-1A PC12 cells. Addition of NE (100 μM) resulted in an initial 3-fold increase in $[Ca^{++}]_i$, which declined quickly to a sustained 2-fold increase over basal. The effect of NE was reversed by the α-adrenergic antagonist phentolamine (10 μM). Administration of the Ca^{++}/ATPase inhibitor TG (10 nM), which depletes intracellular stores by blocking reuptake of Ca^{++}, caused a sustained 3-fold increase in $[Ca^{++}]_i$, which was larger than that caused by NE (Fig. 2A). Treatment with NE caused a large increase in both ERK and JNK phosphorylation in *alpha*-1A-PC12 cells, however neither 1, 3, nor 10 nM TG caused measurable increases in ERK or JNK phosphorylation (Fig. 2B). Figure 3A shows the effect of different concentrations of TG (1–100 nM) on $[Ca^{++}]_i$ in *alpha*-1A-PC12 cells. Although the rate of rise of the Ca^{++} signal increased with increasing concentrations of TG, concentrations between 1 and 100 nM TG all resulted in similar sustained levels of $[Ca^{++}]_i$ (Fig. 3A), and higher concentrations (1000 nM) did not cause a further increase in the sustained level of $[Ca^{++}]_i$ (data not shown). High concentrations of TG (100 and 1000 nM) did cause a small increase in ERK (21 $\pm$ 10% and 16 $\pm$ 6.3% of the response to NE), but not JNK phosphorylation (5 $\pm$ 3% and 3 $\pm$ 3% of NE response) (Fig. 3B).

Combined Effect of TG and NE. After treatment with 10 nM TG for 6 min to deplete intracellular stores of Ca^{++}, addition of NE (100 μM) caused no significant increase in $[Ca^{++}]_i$ levels (4 $\pm$ 5%) in *alpha*-1A-PC12 cells (Fig. 4A). As reported above, 10 nM TG alone caused no significant increase in ERK (2 $\pm$ 1%) or JNK (5 $\pm$ 5%) phosphorylation compared with treatment with NE (100 μM). However, depletion of intracellular stores of Ca^{++} by treatment with 10 nM TG for 6 min did not significantly affect the ability of NE to increase ERK or JNK phosphorylation. When NE was added 6 min after 10 nM TG, increases in ERK and JNK phosphorylation were observed that were similar to or larger than those caused by NE without prior TG treatment (151 $\pm$ 14% and 99 $\pm$ 14% of NE alone, respectively) (Fig. 4B).

Effect of the Ca^{++} Chelator BAPTA. BAPTA AM was used to examine the effects of chelating $[Ca^{++}]_i$ on responses to NE in *alpha*-1A PC12 cells. Cells were preloaded with different concentrations of BAPTA for 30 min before stimulation with NE (100 μM). Increasing concentrations of BAPTA caused a graded loss in the effect of NE on $[Ca^{++}]_i$ (Fig. 5A). Preloading with 10 μM BAPTA AM was sufficient to abolish the NE-induced increase of $[Ca^{++}]_i$, and increasing the BAPTA concentration to 50 μM caused no further effect. The effect of NE on ERK and JNK phosphorylation in the presence of various concentrations of BAPTA is shown in Fig. 5B. Pretreatment with 2 μM, 10 μM, and 50 μM BAPTA AM caused only 31 $\pm$ 23, 24 $\pm$ 19, and 16 $\pm$ 24% reductions in NE-stimulated ERK phosphorylation, respectively. The same concentrations of BAPTA had no significant effect on NE-stimulated JNK phosphorylation, with 2 μM BAPTA causing an 8 $\pm$ 3% decrease and 10 μM and 50 μM BAPTA increasing the effect of NE by 12 $\pm$ 7%, and 40 $\pm$ 27%, respectively (Fig. 5B).

Activation of Pyk2. In *alpha*-1A PC12 cells, NE (100 μM) caused a rapid increase in tyrosine phosphorylation of Pyk2 (Fig. 6A). Chelation of $[Ca^{++}]_i$ with BAPTA (50 μM) caused

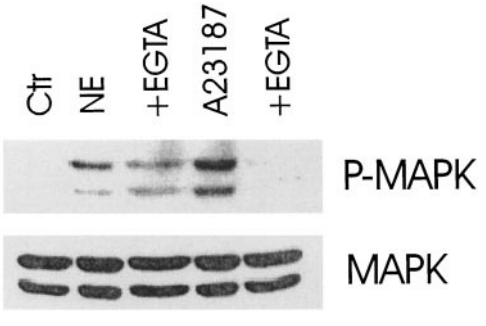

Fig. 1. Effect of norepinephrine and the calcium ionophore A23187 on MAPK phosphorylation in *alpha*-1A-PC12 cells. Western blot analysis of *alpha*-1A-PC12 cells exposed to vehicle (Ctr), 100 μM NE, simultaneous addition of NE and 3 mM EGTA (+EGTA), 10 μM A23187 (A23187), and simultaneous addition of A23187 and 3 mM EGTA (+EGTA). Each lane contained 20 μg of protein. Upper, (Thr-202/Tyr-204) phospho-ERKs (P-MAPK). Lower, Total MAPK. Representative of three experiments.

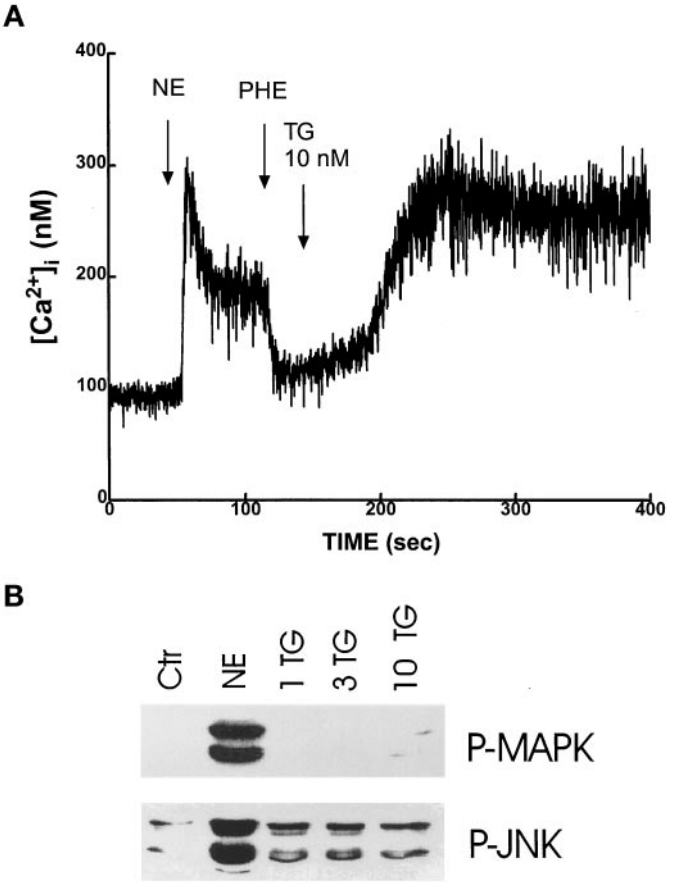

Fig. 2. Effect of TG and NE on $[Ca^{++}]_i$ and MAPK activation in *alpha*-1A-PC12 cells. A, fura-2 loaded cells were exposed to 100 μM NE, 10 μM phentolamine (PHE) and 10 nM TG as indicated by arrows. B, cells were treated for 15 min with vehicle (Ctr), 100 μM NE, 1 nM, 3 nM, or 10 nM TG (1 TG, 3 TG, 10 TG), lysed and Western blot analyses of phospho-ERKs (P-MAPK) and phospho-JNK (P-JNK) run as described. Each lane contained 20 μg protein. Representative of five (A) or two (B) similar experiments.

only a small reduction (30%) in NE-stimulated Pyk2 tyrosine phosphorylation (Fig. 6A). Release of $[Ca^{++}]_i$ by TG (10 nM) did not significantly increase Pyk2 tyrosine phosphorylation (Fig. 6B). Finally, depletion of $[Ca^{++}]_i$ by pretreatment with 10 nM TG for 6 min did not affect the ability of NE to increase Pyk2 tyrosine phosphorylation. In the presence of TG, NE (100 μM) increased tyrosine phosphorylation of Pyk2 ~60% more than was observed with NE alone (Fig. 6B).

Activation of PKC. The role of PKC in ERK and JNK responses to NE in *alpha*-1A PC12 cells was examined by use of the phorbol ester TPA. Cells were exposed to increasing concentrations of TPA in the absence or presence of 10 nM TG to release stored Ca^{++}. Lysis of the cells and Western blot analysis showed that TPA caused a concentration-dependent increase in ERK phosphorylation, with a maximum response similar to that caused by 100 μM NE (Fig. 7). This ERK activation was potentiated by release of $[Ca^{++}]_i$ with 10 nM TG. TPA caused only a small activation of JNK to about 25% of that observed with 100 μM NE (Fig. 7), and this was not increased by the presence of 10 nM TG.

Effect of PKC Inhibition by Bisindolylmaleimide I (GFX 203290). The role of PKC in these responses was further examined by inhibition with the selective PKC inhibitor GFX 203290. GFX caused a concentration-dependent inhibition of TPA-induced activation of both ERKs and JNK with an IC_{50} below 100 nM (Fig. 8). However, concentrations of GFX up to 10 μM had no effect on NE-induced activation of ERKs or JNK in *alpha*-1A-PC12 cells (Fig. 8). In fact, the effect of NE on JNK activation was significantly potentiated at 1 μM GFX. Similar experiments were performed after Ca^{++} chelation with BAPTA. After preloading with 20 μM BAPTA, GFX caused a concentration-dependent inhibition of TPA-induced ERK and JNK activation, but did not diminish the NE-induced activation of ERKs or JNK (data not shown).

P2Y2 Receptor Activation. We examined whether these results were applicable to other $G_{q/11}$-coupled receptors by studying the effects of ATP and UTP on ERK activation. Soltoff et al. (1998) have reported that PC12 cells express endogenous P2Y2 receptors that are activated equipotently by UTP and ATP, and cause activation of ERKs. We found that both UTP and ATP (100 μM) caused activation of ERKs similar to that caused by NE and NGF in our isopropyl

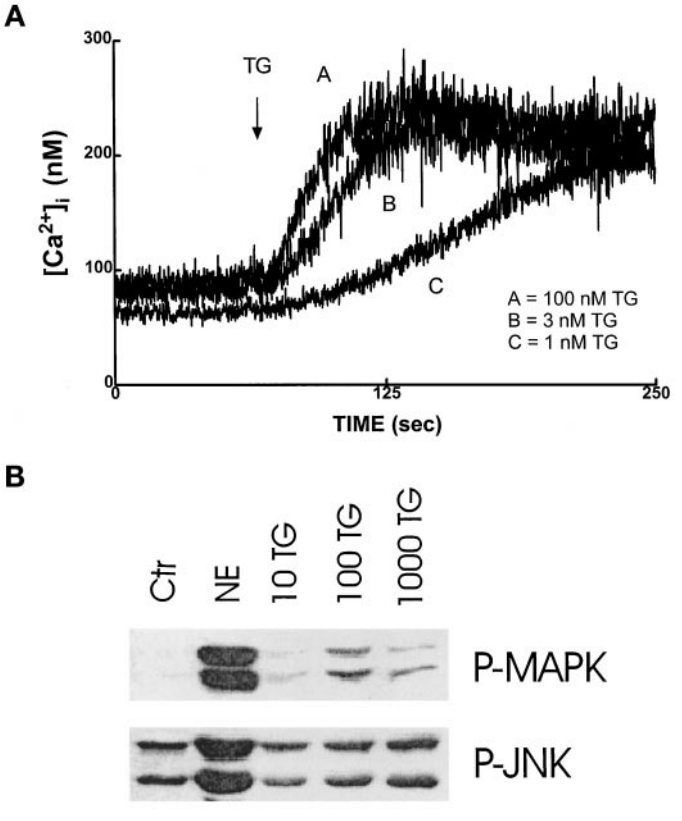

Fig. 3. Effect of different concentrations of TG on $[Ca^{++}]_i$ and MAPK activation in *alpha*-1A-PC12 cells. A, fura-2 loaded cells were exposed to 1 nM, 3 nM, or 100 nM TG as indicated. B, cells were treated 15 min with vehicle (Ctr), 100 μM NE, 10 nM, 100 nM, or 1000 nM TG (10 TG, 100 TG, 1000 TG), lysed, and Western blot analysis of phospho-ERKs (P-MAPK) and phospho-JNK (P-JNK) was performed as described. Each lane contained 20 μg protein. Representative of four (A) and three (B) similar experiments.

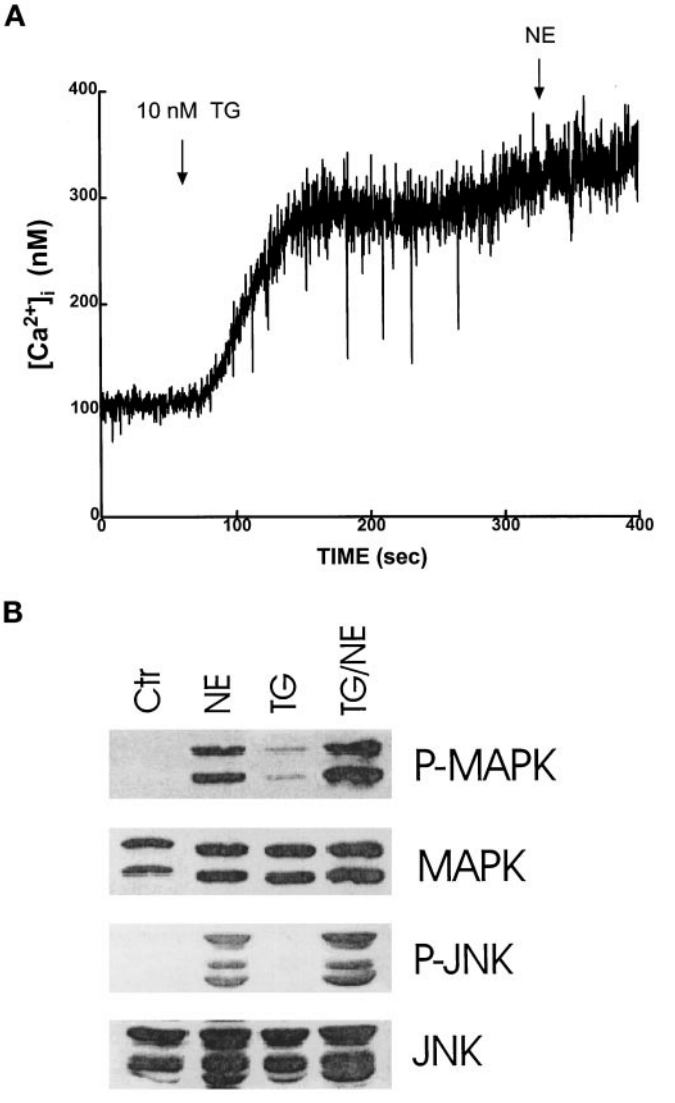

Fig. 4. Effect of prior TG treatment on $[Ca^{++}]_i$ and MAPK responses to NE in *alpha*-1A-PC12 cells. A, fura-2 loaded cells were exposed to 10 nM TG and 100 μM NE as indicated. B, cells were treated for 15 min with vehicle (Ctr), 100 μM NE alone (NE), 21 min with 10 nM TG alone (TG), or 6 min with 10 nM TG and then a further 15 min with 100 μM NE in the continued presence of TG (TG/NE), lysed, and Western blot analyses of phospho-ERKs (P-MAPK) and phospho-JNK (P-JNK) run as described. Each lane contained 20 μg protein. Representative of five (A) and four (B) similar experiments.

β-D-thiogalactoside-stimulated *alpha*-1A-PC12 cells (Fig. 9), confirming the presence of endogenous P2Y2 receptors. Similar to our results with *alpha*-1A ARs, the effect of activation of P2Y2 receptors by UTP was not blocked by preloading with 20 μM BAPTA AM or inhibition with 2 μM GFX (Fig. 9).

Discussion

Previous studies have suggested that Ca^{++} may play an important role in MAPK responses to GPCRs in many cell types. ERK and JNK activation requires Ca^{++} in some cell types (Chao et al., 1992; Rosen et al., 1994; Mitchell et al., 1995; Zohn et al., 1995) but not others (Cadwallader et al., 1997). Specific Ca^{++}-sensitive enzymes have been implicated in certain responses (Daulhac et al., 1997; Enslen et al., 1996; Scimeca et al., 1997). In PC12 cells, depolarization-evoked opening of voltage-gated Ca^{++} channels or treatment with a Ca^{++} ionophore activates growth factor-mediated signaling pathways (Rosen et al., 1994; Rosen and Greenberg, 1996). ERKs are also activated by bradykinin and lysophosphatidic acid acting through GPCRs in PC12 cells, and this has been suggested to involve Ca^{++}-dependent activation of the tyrosine kinases Pyk2 and Src (Dikic et al., 1996; Lev et al., 1995). However, no direct activation of these enzymes by Ca^{++} has been observed in vitro.

We showed previously that activation of *alpha*-1A ARs by NE activates ERKs, JNK, and p38 MAPK in transfected PC12 cells, and causes them to differentiate to a neuronal-like phenotype similar to that caused by NGF (Williams et al., 1998). We used these cells to examine the mechanisms by which $G_{q/11}$-coupled receptors activate MAPK pathways. We focused specifically on whether $[Ca^{++}]_i$ mobilization and PKC activation, the major signals generated by receptor activation, are important to these responses. We concentrated on activation of ERKs and JNK, because these responses are more robust than p38 MAPK activation (Williams et al., 1998).

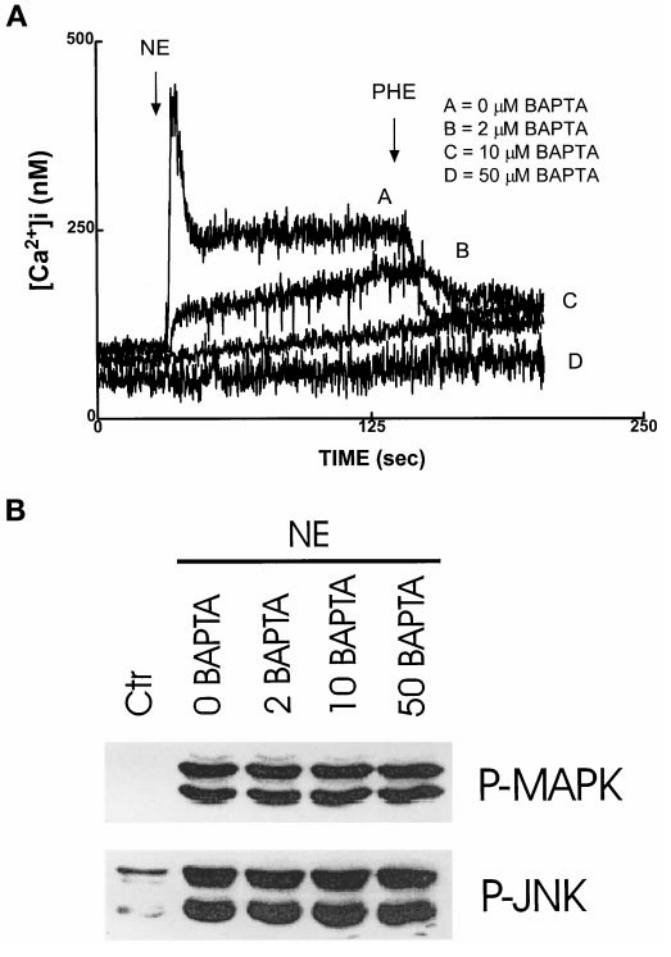

Fig. 5. Effect of Ca^{++} chelation with BAPTA on $[Ca^{++}]_i$ and MAPK responses to NE in *alpha*-1A-PC12 cells. A, fura-2 loaded cells were exposed to 100 μM NE and 10 μM phentolamine (PHE) after preloading for 30 min with the indicated concentrations of BAPTA AM. B, cells were treated 30 min with vehicle (Ctr), or 0, 2, 10, or 50 μM BAPTA AM (0, 2, 10, and 50 BAPTA) before 15 min stimulation with 100 μM NE, lysed, and Western blot analyses of phospho-ERKs (P-MAPK) and phospho-JNK (P-JNK) run as described. Each lane contained 20 μg protein. Representative of four (A) and three (B) similar experiments.

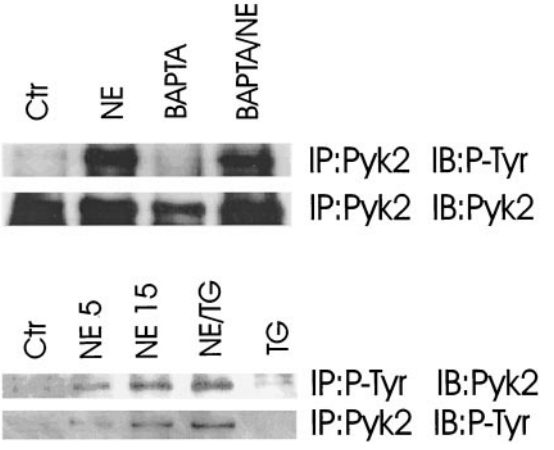

Fig. 6. Effect of NE, BAPTA, and TG on tyrosine phosphorylation of Pyk2 in *alpha*-1A-PC12 cells. (Upper) Cells were exposed to vehicle (Ctr), 100 μM NE, preloaded 30 min with 50 μM BAPTA (BAPTA), or preloaded 30 min with 50 μM BAPTA and treated with NE (BAPTA/NE). Upper lane: immunoprecipitation (IP) with anti-Pyk2 antibody and immunoblot (IB) with anti-phosphotyrosine (P-Tyr) antibody. Lower lane: IP with anti-Pyk2 antibody and IB with the same antibody. Lower panel: cells were treated with 100 μM NE for 5 min (NE 5) or 15 min (NE 15) or 10 nM TG for 6 min before 100 μM NE for 15 min (NE/TG), or 10 nM TG alone (TG). Upper lane: IP with anti-P-Tyr antibody and IB with anti-Pyk2 antibody. Lower lane: IP with anti-Pyk2 antibody and IB with anti-P-Tyr antibody.

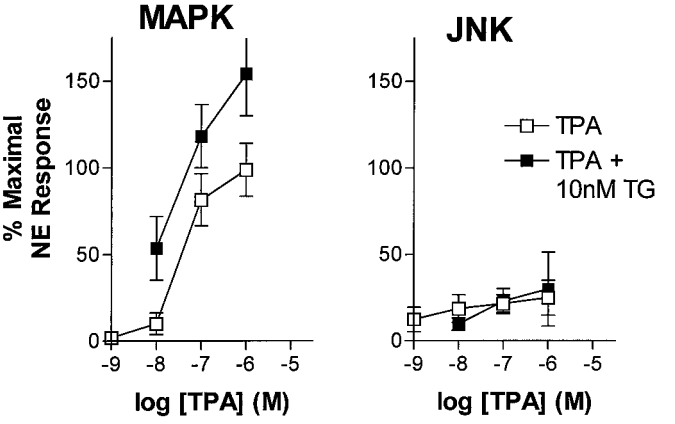

Fig. 7. Effect of TPA or TPA plus TG on MAPK responses in *alpha*-1A-PC12 cells. Cells were exposed to the indicated concentrations of TPA in the absence (open symbols) or presence (solid symbols) of 10 nM TG for 15 min, lysed, and Western blot analyses of phospho-ERKs (MAPK) and phospho-JNK (JNK) run as described. Each lane contained 20 μg of protein. Each point is the mean ± S.E.M. of data from three to five experiments.

Treatment with a Ca^{++} ionophore has been shown to stimulate ERK and Pyk2 phosphorylation in PC12 cells (Lev et al., 1995), and we confirmed this in our cells. A23187 increased ERK phosphorylation to a greater extent than did NE. However, we used the Ca^{++}/ATPase inhibitor TG (Jackson et al., 1988) to more closely mimic release of stored Ca^{++} by GPCR activation. We found that increases in $[Ca^{++}]_i$ caused by TG equal to or greater than those caused by NE did not significantly increase ERK or JNK phosphorylation. Lower concentrations of TG caused a slower release of Ca^{++}, although the plateau Ca^{++} level was similar for all TG concentrations. TG has been shown to increase ERK activity in other cells (Chao et al., 1992; Fleming et al., 1995; Romanelli and van de Werve, 1997; Daulhac et al., 1997) although the effect is usually small. The effect of TG on ERK activation is blocked by a dominant negative mutant of Raf (Chao et al., 1994). We also found that high concentrations of TG caused small effects on ERK, but not JNK, phosphorylation, although these concentrations were significantly higher than those needed to mimic the response to NE.

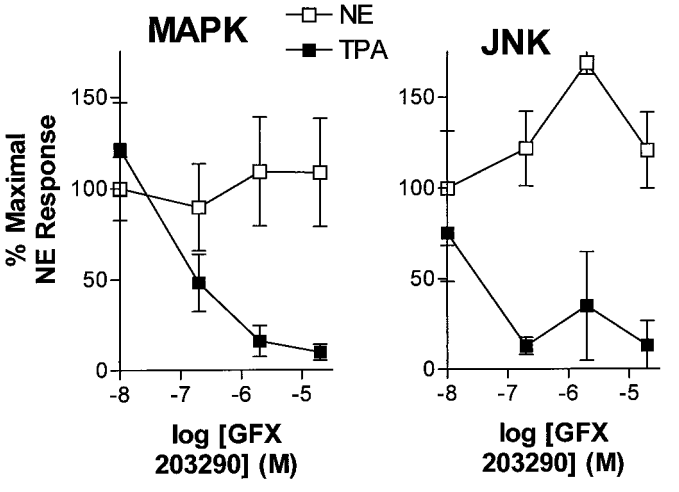

Fig. 8. Effect of the PKC inhibitor GFX 203290 on NE- and TPA-induced MAPK responses in *alpha*-1A-PC12 cells. Cells were exposed to 100 μM NE or 1000 nM TPA in the presence of the indicated concentrations of GFX for 15 min, lysed, and Western blot analyses of phospho-ERKs (MAPK) and phospho-JNK (JNK) run as described. Each lane contained 20 μg of protein. Each point is the mean ± S.E.M. of data from three to five experiments.

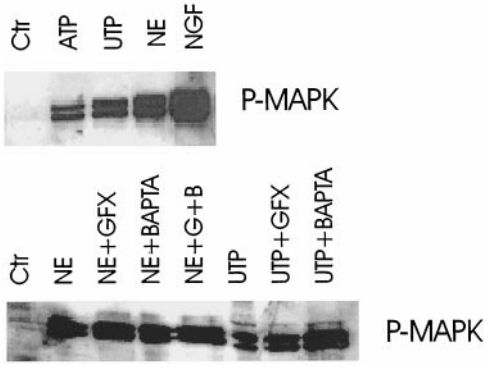

Fig. 9. Effect of purinergic agonists on MAPK phosphorylation in *alpha*-1A-PC12 cells. (Upper) Western blots of *alpha*-1A-PC12 cells exposed to vehicle (Ctr), 100 μM ATP, 100 μM UTP, 100 μM NE, or 50 ng/ml NGF. Gel was blotted with antibody specific for activated, dually phosphorylated ERKs (P-MAPK). (Lower) Effect of pretreatment with 20 μM BAPTA or 2 μM GFX or both (+G+B) on responses to NE (100 μM) or UTP (100 μM). Each lane contained 10 μg of protein. Representative of two experiments.

TG was also used to empty $[Ca^{++}]_i$ stores, to determine whether NE could cause ERK or JNK activation in the absence of a significant component of Ca^{++} release. This would provide evidence that other signals are required for the observed responses. After 6-min exposure to TG, addition of NE did not further increase $[Ca^{++}]_i$ but still caused a robust activation of ERKs and JNK. This activation was indistinguishable from that caused by NE without TG pretreatment, strongly suggesting that the Ca^{++} release associated with *alpha*-1A AR activation is not primarily responsible for ERK or JNK activation. Previously, Daulhac et al. (1997) showed that depletion of $[Ca^{++}]_i$ with a high concentration of TG (1 μM) in pancreatic islet tumor cells inhibited gastrin-mediated activation of ERKs, suggesting that these signaling pathways may be cell specific. Our results show that Ca^{++} mobilization is not required for NE-stimulated ERK or JNK activation in *alpha*-1A-PC12 cells, although they do not rule out the possibility that an increased basal Ca^{++} is important.

This possibility was examined by preloading *alpha*-1A-PC12 cells with the Ca^{++} chelator BAPTA. Concentration-response relationships showed that preloading with 10 μM BAPTA was sufficient to abolish NE-induced increases in cytoplasmic Ca^{++}. However, preloading with up to 50 μM BAPTA did not significantly inhibit NE-stimulated ERK or JNK phosphorylation, clearly demonstrating that the increase in $[Ca^{++}]_i$ caused by NE in *alpha*-1A-PC12 cells is not necessary for ERK or JNK activation.

The tyrosine kinase Pyk2 has been implicated as an important mediator of ERK activation by GPCRs in PC12 cells (Dikic et al., 1996; Lev et al., 1995). In *alpha*-1A-PC12 cells, NE caused a large and rapid increase in tyrosine phosphorylation of Pyk2 similar to that observed previously with bradykinin. However, TG did not increase Pyk2 phosphorylation and depletion of $[Ca^{++}]_i$ with TG did not block NE-stimulated Pyk2 phosphorylation. Finally, chelation of $[Ca^{++}]_i$ with BAPTA did not substantially decrease NE-induced Pyk2 tyrosine phosphorylation, suggesting that this response is not mediated by increased $[Ca^{++}]_i$. Similar results have been reported recently with GPCR chemokine receptors. Chemokines and HIV-1 envelope glycoproteins rapidly induce tyrosine phosphorylation of Pyk2 without increasing $[Ca^{++}]_i$ (Davis et al., 1997) supporting the hypothesis that GPCRs may activate Pyk2 through a Ca^{++}-independent mechanism.

Ca^{++} is not the only second messenger formed in response to activation of $G_{q/11}$-linked receptors. Diacylglycerol is released after phospholipase C activation and acts synergistically with Ca^{++} to activate PKC (Berridge and Irvine, 1984). Activation of PKC can activate ERKs in nearly all cells examined (Downward et al., 1990; Kolch et al., 1993; Van Renterghem et al., 1994; Seger et al., 1995; Soltoff, 1998; Sozeri et al., 1992; Yamaguchi et al., 1995; Young et al., 1996); and we obtained similar results in our *alpha*-1A-PC12 cells. The phorbol ester TPA caused a concentration-dependent activation of ERKs similar to that caused by NE, although TPA caused a much smaller JNK activation than did NE. The specific PKC inhibitor GFX (Gekeler et al., 1996) inhibited the response to TPA as expected. However, concentrations of GFX much greater than those required to inhibit the TPA response had no effect on NE-mediated activation of

ERKs or JNK in *alpha*-1A-PC12 cells. This suggests that PKC does not mediate the effect of NE on MAPK responses in PC12 cells.

We also examined whether MAPK responses to NE in these cells require simultaneous activation of both the Ca^{++} and PKC arms of the pathways. Release of Ca^{++} stores by TG potentiated the effect of PKC activation by TPA on ERK, but not on JNK, activation. Chelation of $[Ca^{++}]_i$ by BAPTA did not alter these results. After preloading with BAPTA, GFX still inhibited activation of ERKs and JNK by TPA but not by NE, even at GFX concentrations up to 10 μM (data not shown). Thus, simultaneous inhibition of increases in $[Ca^{++}]_i$ by BAPTA and inhibition of PKC activation by GFX did not alter NE-stimulated ERK and JNK activation in *alpha*-1A-PC12 cells.

To determine whether these results were also true for other GPCRs, we studied the purinergic receptors expressed endogenously in PC12 cells. Nucleotide triphosphates increase Ca^{++} and activate MAPK in PC12 cells. In some PC12 subclones these responses are mediated by $G_{q/11}$-coupled P2Y2 receptors responsive to both UTP and ATP (Soltoff, 1998; Soltoff et al., 1998), whereas in others they are mediated by ionotropic P2X receptors responsive only to ATP (Swanson et al., 1998). Our PC12 cells express $G_{q/11}$-coupled P2Y2 receptors similar to those of Soltoff et al. (1998), because they respond to both UTP and ATP. MAPK responses to UTP in these cells were not blocked by chelation of $[Ca^{++}]_i$ with BAPTA or inhibition of PKC by GFX, suggesting that our results with *alpha*-1A ARs can be generalized to other receptors of the same family. Soltoff (1998) found that a combination of BAPTA plus extracellular EGTA, or overnight depletion of PKC by phorbol ester pretreatment, partially reduced UTP-induced activation of MAPK in PC12 cells, and concluded that Ca^{++} and PKC may play important roles in P2Y2-mediated MAPK activation. The different results obtained here are probably due to the use of less harsh treatments (BAPTA alone, GFX inhibition) to accomplish the same ends. Because we independently confirmed that each inhibitor had its desired effect, we believe our results support the conclusion that mitogenic responses to $G_{q/11}$-coupled receptors in PC12 cells may be independent of traditional second messenger pathways.

Overall, these results show that neither the increase in $[Ca^{++}]_i$ nor the activation of PKC caused by NE in *alpha*-1A-PC12 cells is critical for MAPK responses. They also suggest that tyrosine phosphorylation of Pyk2 in response to GPCR activation can occur through a Ca^{++}-independent mechanism. Because the traditional second messenger pathways activated by *alpha*-1A ARs do not mediate either ERK or JNK activation in PC12 cells, it will be important to further define the signals generated by these receptors to activate mitogenic responses in this cell type.

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