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Pertussis Toxin Inhibits Norepinephrine-Stimulated Inositol Phosphate Formation in Primary Brain Cell Cultures

KAREN M. WILSON and KENNETH P. MINNEMAN

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

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SUMMARY

Norepinephrine (NE) increased formation of [^{3}H]inositol phosphates ([^{3}H]InsPs) in primary cultures of neuronal and glial cells from 1-day-old rat brain. This response appeared to be mediated by α_1-adrenergic receptors, because prazosin was 40-fold more potent than yohimbine in blocking it. Pretreatment with pertussis toxin (PTX) dose-dependently decreased this response by 70–80%. The IC_{50} for PTX (7 ng/ml) was similar to that for blocking of α_2-adrenergic receptor-mediated decreases in cyclic AMP accumulation in the same cells. PTX pretreatment caused only a small, not statistically significant, inhibition of the [^{3}H]InsP response to the muscarinic cholinergic receptor agonist carbachol in these cells. Radioligand binding studies showed that both neuronal and glial cultures contained mixed populations of α_{1a}- and α_{1b}-adrenergic receptor subtypes. Selective inactivation of the α_{1b} population by chloroethylclonidine reduced NE-stimulated [^{3}H]InsP formation by 25 ± 6%. Pretreatment with both PTX and chloroethylclonidine caused additive decreases (90 ± 3%) in the NE response. NE-stimulated [^{3}H]InsP formation was partially dependent on extracellular calcium, because it was decreased 64 ± 6% by removal of calcium and 56 ± 13% by addition of 1 mM $CdCl_2$, although it was not affected by 1 μM nifedipine. These results suggest that NE stimulates [^{3}H]InsP formation in neuronal and glial cultures through a pertussis toxin-sensitive guanine nucleotide-binding protein. This response appears to be mediated primarily by the α_{1a} subtype and may be subsequent to calcium influx.

α_1-Adrenergic receptors are thought to belong to the large family of receptors that initiate their cellular signals by activating phospholipase C (1). This enzyme cleaves PIP_2 into Ins(1,4,5)P_3 and diacylglycerol, which release stored intracellular Ca^{2+} and activate protein kinase C, respectively (2). Although the mechanisms by which receptor activation increases phospholipase C activity are not yet clear, G proteins appear to play an important role in this coupling (3, 4). Guanine nucleotides modulate the affinities of agonists for many phospholipase C-coupled receptors in membranes (3–5), suggesting that these receptors couple to G proteins. Guanine nucleotides also stimulate formation of InsPs in membrane preparations from a variety of cells (3, 4, 6–8), apparently by direct G protein activation.

However, the G protein(s) responsible for coupling receptors to phospholipase C have not yet been identified. In some cells, pretreatment with PTX markedly reduces increases in InsP formation caused by stimulation of a variety of different hormone and neurotransmitter receptors (9–12). PTX is known to block receptor-mediated inhibition of adenylate cyclase and inactivate at least four separate G proteins (G_o, G_{i1}, G_{i2}, and G_{i3}) by ADP-ribosylation (13). However, in many cells PTX pretreatment does not affect receptor-mediated increases in InsP formation (12, 14–18). Because PTX blocks InsP responses in some cells but not others, it is likely that two distinct G proteins are involved in coupling receptors to phospholipase C (4, 12). Whether these two different G proteins are activated by the same receptors in different cells or by distinct but closely related receptor subtypes is not yet clear.

Similar heterogeneity is observed with α_1-adrenergic receptors. Guanine nucleotides modulate agonist affinity at α_1-adrenergic receptor binding sites (5, 19, 20). An early study in fat cells showed that PTX pretreatment blocked α_1 receptor-stimulated ^{32}P incorporation into phosphatidylinositol (21). However, PTX pretreatment has generally been found to have no effect on α_1-receptor-stimulated InsP formation in most cells (16, 22–26) or on positive chronotropic (27), inotropic (28), or cyclic AMP (29) responses in heart. Conversely, some α_1-mediated responses, such as negative chronotropic effects in cardiac cell cultures (27), activation of phospholipase A_2 (25, 30), and calcium-dependent pressor responses (31), have been found to be blocked by PTX.

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ABBREVIATIONS: PIP_2, phosphatidyl inositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; DMEM-C, Dulbecco's modified Eagles medium containing 10% calf bovine serum; ARC, cytosine arabinoside; ^{125}I-BE, ^{125}I-BE 2254; KRB, Krebs Ringer bicarbonate buffer; PBS, phosphate-buffered saline; G protein, guanine nucleotide-binding regulatory protein; PTX, pertussis toxin; InsP, inositol phosphate; CEC, chloroethylclonidine; NE, norepinephrine; Li-KRB, lithium-Krebs Ringer bicarbonate buffer; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

There is now strong evidence for at least two pharmacologically distinct subtypes of α_1-adrenergic receptors (32–34). Han *et al.* (33) proposed that the α_{1b} subtype controlled release of stored intracellular Ca^{2+} by an $Ins(1,4,5)P_3$ mechanism, whereas the α_{1a} subtype selectively controlled influx of extracellular Ca^{2+}. However, the signaling systems utilized by these two subtypes have yet to be completely clarified (35). In the brain, α_{1a} and α_{1b} receptors are heterogeneously distributed, but there is no correlation between either subtype and the InsP response to NE (36). We recently showed that both subtypes can stimulate formation of InsPs,[1] possibly with differential involvement of Ca^{2+} influx.[2] Here we describe the effect of pertussis toxin and Ca^{2+} on InsP responses to NE in primary cultures of neuronal and glial cells from rat brain.[3]

Experimental Procedures

Materials. The following materials were used: (−)-NE bitartrate, yohimbine, forskolin, carbachol, ARC, deoxyribonuclease, poly-L-lysine (M_r 150,000), amphotericin B, and penicillin (Sigma, St. Louis, MO); calf bovine serum and DMEM and calf serum (HyClone Laboratories, Logan, UT); 1× crystallized trypsin (150 units/mg) (Worthington Biochemicals, Freehold, NJ); phenoxybenzamine (Smith, Kline and French, Philadelphia, PA); UK 14304 (5-bromo-6-[2-imiolazolin-2-ylamino]-quinoxaline) and prazosin (Pfizer, Sandwich, Kent, England); PTX (List Biological Laboratories, Campbell, CA); BE 2254 [(2-β-(4-hydroxyphenyl)ethylaminomethyl)-tetralone] (Beiersdorf, Hamburg, FRG); $Na^{125}I$ (Amersham, Arlington Heights, IL); [^{3}H]inositol (20–40 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO); and [2,8-^{3}H]adenine and [2,8-^{3}H]cAMP (New England Nuclear).

Preparation of cells. Primary cultures of neuronal and glial cells were prepared from 1-day-old rat brains essentially by the method of Raizada (37), as described previously (38).[3] Brains were removed and placed in an isotonic salt solution (1 liter) containing 0.25 μg of amphotericin B, 100 μg of streptomycin, and 100 units of penicillin, pH 7.2. Pia mater and blood vessels were removed, and the brains were minced with scissors (2-mm sections). Tissue from 7–14 brains was suspended in 25 ml of 0.25% trypsin (w/v) in an isotonic salt solution and placed in a 37° shaking water bath for 6 min. Deoxyribonuclease I (160 μg) was then added to the cell suspension and the incubation was continued for an additional 6 min. Dissociated cells were collected in 10 ml of DMEM-C. Undissociated tissue was triturated several times and all cells were washed with 90 ml of DMEM-C and centrifuged for 10 min at 1000 × *g*. Recoveries averaged 40–50 × 10^6 cells/brain. Cells were resuspended in DMEM-C and plated in Falcon dishes precoated with poly-L-lysine. For neuronal cultures, 1.5 × 10^6 cells/ml were placed on 35-mm dishes (2 ml, [^{3}H]InsP assays) or 60-mm dishes (4 ml, binding assays). Cells were incubated at 37° in a humidified incubator with 5% CO_2/95% air. After 3 days, cells were treated with 10 μM ARC. This treatment inhibits the multiplication of cells, the majority which are nonneuronal in origin. After 48 hr, the ARC medium was replaced with fresh DMEM-C. Cells were incubated an additional 2 to 10 days before being used for binding experiments. For measurement of InsPs, cells were incubated with [^{3}H]inositol starting 2–4 days after ARC treatment.

Glial cells were plated at 2 × 10^6 cells/ml (4 ml) in 60-mm dishes and not treated with ARC. After cells reached confluency (7 days), they were dissociated from the dishes with trypsin (0.25%), washed with DMEM-C, and centrifuged at 1000 × *g* for 10 min. Cells were resuspended at a density of 0.5 × 10^6/ml in DMEM-C and plated in either 35-mm dishes (2 ml, [^{3}H]InsP assays) or 60-mm dishes (4 ml, binding assays). Glial cells were prelabeled with [^{3}H]inositol starting 2–4 days after trypsinization and allowed to grow to confluency (7–21 days) before experimentation.

Cells were examined microscopically before use. Glial cultures showed >95% staining for glial fibrillary acidic protein, suggesting that they are mainly astrocytic with a few oligodendrocytes (37). Neurons generally showed 5–20% staining for glial fibrillary acidic protein, showing a small cross-contamination with glial cells. Neurons showed 70–80% staining with neuron-specific enolase (37).

^{125}I-BE binding. BE 2254 was radioiodinated to theoretical specific activity (39) and stored in methanol at −20°. The specific binding of ^{125}I-BE to α_1-adrenergic receptors was determined in membranes prepared from neurons and glial cells. Cells were washed with PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6), scraped from the dishes with the aid of a rubber policeman, and centrifuged at 1000 × *g* for 10 min. Cells were resuspended in PBS, homogenized with a Polytron, and centrifuged at 30,000 × *g* for 30 min. The membrane pellet was resuspended in PBS to a protein concentration of approximately 0.1 mg/ml (neurons) or 0.3 mg/ml (glia). Specific ^{125}I-BE binding was measured by incubation of 0.1 ml of cell preparation with ^{125}I-BE in PBS, in the presence or absence of competing drugs, in a final volume of 0.25 ml for 20 min at 37°, as described previously (40). The incubation was stopped by addition of 10 ml of 10 mM Tris·HCl (pH 7.4) and filtration over glass fiber filters (Schleicher and Schuell no. 30) under vacuum. Filters were washed with an additional 10 ml of buffer and dried and radioactivity was determined in a γ-counter. Nonreceptor binding was determined in the presence of 10 μM phentolamine. Saturation curves were determined by incubation of tissue with six increasing concentrations of ^{125}I-BE (25–800 pM), and data were analyzed by Scatchard analysis. Protein content was determined by the Bradford method (41). The potency of WB 4101 in competing for specific ^{125}I-BE binding sites was determined by incubation of a single concentration of ^{125}I-BE (40–50 pM) in either the presence or absence of 15 concentrations of the competing drug (3 × 10^{-11} to 1.8 × 10^{-7} M). IC_{50} values were determined from the *x*-intercept of a Hill plot and correlated to K_i values. The best two-site fit for a binding curve was calculated by minimizing the sum of squares of the errors using nonlinear regression analysis.

Measurement of [^{3}H]inositol metabolism. Accumulation of [^{3}H]InsPs was measured in the presence of LiCl, as described previously.[3] Cells were prelabeled with [^{3}H]inositol (1 μCi/dish) for 10 days unless otherwise specified. Cells were then washed three times with Li-KRB (in mM: 10 LiCl, 110 NaCl, 5.5 KCl, 2.5 $CaCl_2$, 20 $NaHCO_3$, 11 glucose, 0.029 $CaNa_2EGTA$, 1.2 $MgCl_2$, and 1.2 $Na_2H_2PO_4$). Li-KRB (1 ml) was added to each plate, and cells were incubated with or without drugs (in 10 μM ascorbic acid) for 1 hr at 37°. The incubation medium was then aspirated, and 0.66 ml of ice-cold methanol was added. Cells were scraped from the dishes with a rubber policeman and added to a tube containing 0.66 ml of chloroform. Dishes were washed with 0.66 ml of distilled H_2O, which was added to the cell suspension. Samples were sonicated for 10 sec and centrifuged at 10,000 × *g* for 5 min to separate aqueous and organic phases.

Aliquots of the aqueous phase (0.75 ml) were added to prepared Dowex columns [13 mm of DOWEX AG 1 × 8-200 anion exchange resin (formate form) in 8 × 200 mm Kontes columns], which previously had been washed with 40 ml of 10 mM Tris-formate buffer (pH 7.4). Columns were washed with 40 ml of 5 mM *myo*-inositol, and [^{3}H]InsPs were eluted with 1 ml of 1 M ammonium formate/0.1 N formic acid. Three milliliters of Scintiverse (Fisher) were added and samples were shaken vigorously and counted with 40% efficiency. Columns were regenerated with 4 ml of 1.0 N formic acid and stored in 0.1 N formic acid. Aliquots of the organic phase (200 μl) were allowed to evaporate overnight. Water (0.3 ml) and scintillation fluid (2 ml) were then added and samples were counted to determine total [^{3}H]inositol incorporation.

Measurement of cyclic AMP accumulation. Increases in cAMP

[1] C. Han, K. M. Wilson, and K. P. Minneman. α_1-Adrenergic receptor subtypes and formation of inositol phosphates in dispersed hepatocytes and renal cells. *Mol. Pharmacol.* **37**:903–910 (1990).

[2] K. M. Wilson and K. P. Minneman, unpublished results.

[3] K. M. Wilson, S. Gilchrist, and K. P. Minneman. Comparison of α_1-adrenergic receptor-stimulated inositol phosphate formation in primary neuronal and glial cultures. *J. Neurochem.*, in press.

accumulation were determined by the [^{3}H]adenine prelabeling method (42) modified for cell cultures. Glial cells were grown to confluency and 1 μl [^{3}H]adenine (1 μCi) was added to each dish and incubated at 37° for 2 hr. Cells were then washed three times with KRB (in mM: 120 NaCl, 5.5 KCl, 2.5 $CaCl_2$, 20 $NaHCO_3$, 11 glucose, 0.029 $CaNa_2$ EGTA, 1.2 $MgCl_2$, and 1.2 NaH_2PO_4). Drugs (forskolin, UK 14304) were then added to 1 ml of KRB and incubated for 10 min at 37°. The reaction was terminated by the addition of 100 μl of 77% trichloroacetic acid. An aliquot (50 μl) of 10 mM cAMP was added to each dish as a carrier. Cells were scraped from dishes with the aid of a rubber policeman and placed in centrifuge tubes. Dishes were washed with an additional 0.5 ml of KRB, which was added to the tubes. Cells were homogenized with a Polytron and a 50-μl aliquot was removed for determination of total tritium incorporation. The tubes were centrifuged for 15 min at 20,000 × *g*. The [^{3}H]cAMP formed was isolated from the supernatant by sequential Dowex and alumina chromatography. Results were expressed as percentages of the total radioactivity incorporated into the cells that was converted to [^{3}H]cAMP (percentage of conversion).

Data analysis. All InsP data were determined by calculation of the amount of [^{3}H]InsP formed divided by total [^{3}H]inositol incorporated into lipid (cpm/total cpm incorporated). Dose-response curves were analyzed by linear regression of all points between 20 and 80% of maximal response. Analysis of variance and the Student's paired and unpaired *t* tests were used to test significance, and *p* values less than 0.05 were considered significant. All data are expressed as mean ± standard error.

Results

Effect of PTX on NE-stimulated [^{3}H]InsP formation. Treatment with 100 μM NE for 1 h increased [^{3}H]InsP formation 4.3-fold in glia and 2.5-fold in neurons (Fig. 1). Pretreatment of the cells with 300 ng/ml PTX for 15–20 hr significantly reduced NE stimulation by 75 ± 2% in glial cultures and 70 ± 13% in neuronal cultures (Fig. 1). Basal [^{3}H]InsP accumulation in the absence of NE was higher and the percentage of stimulation by NE was lower in neurons than glial cells. Therefore, further studies were conducted primarily in glial cultures.

Potency of PTX. The potency of PTX in decreasing NE-stimulated [^{3}H]InsP formation was determined in glial cultures (Fig. 2). The IC_{50} for PTX was 7 ± 5.4 ng/ml (four experiments), and maximum inhibition (83%) was observed with 100 ng/ml PTX. For comparison, the potency of PTX in blocking α_2-adrenergic receptor-mediated inhibition of cAMP accumulation was examined in the same cells. The α_2-agonist UK 14,304 (10 μM) decreased forskolin-stimulated cAMP accumulation (Fig. 3), and this effect was reversed by PTX with an IC_{50} of 19 ± 11 ng/ml (three experiments), similar to that for inhibition of the [^{3}H]InsP response.

Inhibition by prazosin and yohimbine. The potencies of the competitive antagonists prazosin (α_1-selective) and yohimbine (α_2-selective) in inhibiting NE-stimulated [^{3}H]InsP accumulation were determined in glial cultures. Prazosin (IC_{50} = 0.01 μM) was 40-fold more potent than yohimbine (IC_{50} = 3.9 μM) in blocking the response to 100 μM NE (Fig. 4), indicating that this response is mediated primarily by α_1-adrenergic receptors.

Effect of PTX on carbachol stimulation. The effect of PTX pretreatment on the [^{3}H]InsP response to the muscarinic cholinergic receptor agonist carbachol was examined. A 1-hr incubation with NE (100 μM) or carbachol (1 mM) caused similar increases in [^{3}H]InsP accumulation, 3.8 ± 0.3- and 3.4 ± 0.4-fold, respectively (Fig. 5). Pretreatment with 300 ng/ml PTX significantly reduced the response to NE to 1.8 ± 0.4-fold

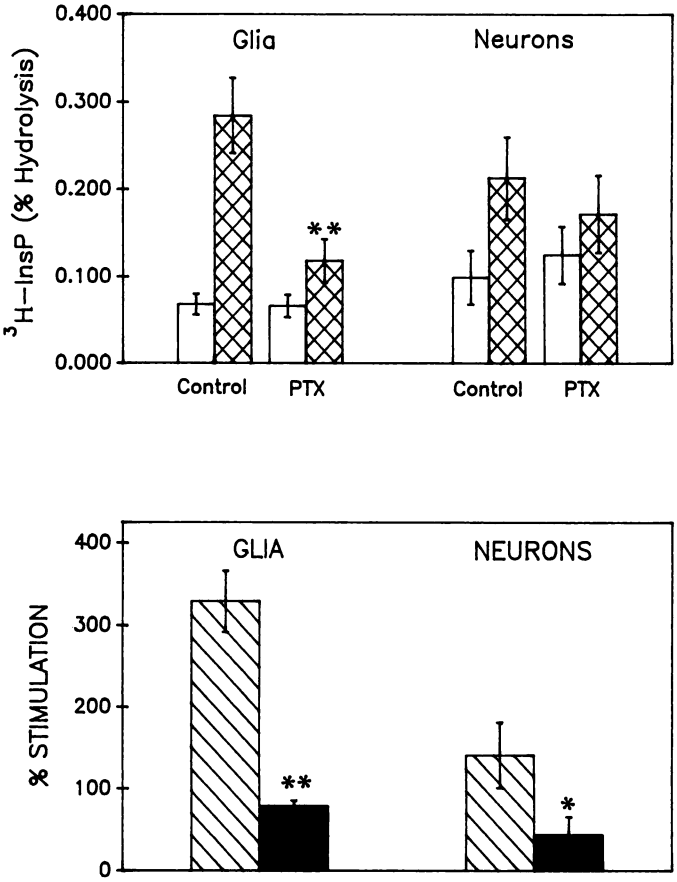

Fig. 1. Effect of PTX on NE-stimulated [^{3}H]InsP formation in neuronal and glial cultures. Cells were pretreated for 15–20 hr with 300 ng/ml PTX, washed, and then incubated with 100 μM NE for 1 hr. *Upper*, □, basal levels; ■, levels in the presence of NE. *Lower*, percentage of NE stimulation over basal in control (▨) and PTX-treated cells (■). Each *bar* represents the mean ± standard error of triplicate determinations from three experiments. *, $p < 0.05$; **, $p < 0.001$.

but caused a smaller decrease in the response to carbachol (to 2.6 ± 0.3-fold), which was not statisticaly significant. Similar results were also obtained in neurons (data not shown).

Determination of α_{1a}- and α_{1b}-adrenergic receptor subtypes. Radioligand binding measurements were utilized to determine the relative proportion of α_1-adrenergic receptor subtypes in these cultures. α_{1a} and α_{1b} subtypes were differentiated by their sensitivity to alkylation by CEC and their affinity for the competitive antagonist WB 4101. Scatchard analysis of ^{125}I-BE 2254 binding in cells pretreated with or without CEC showed mixed population of α_{1a} and α_{1b} subtypes in both neuronal and glial cultures (Table 1). Hill coefficients for WB 4101 inhibition of ^{125}I-BE binding were less than 1 (Table 2) and nonlinear regression showed that WB 4101 inhibition curves were better fit by a two-site model in both neurons and glial cells (Table 2). The proportion of ^{125}I-BE binding sites that were sensitive to CEC alkylation was similar to the proportion with a low affinity for WB 4101 in both cell types (Tables 1 and 2), as shown previously in many other tissues (34, 36), demonstrating the existence of both α_{1a} and α_{1b} subtypes in these cultures.

Effect of CEC on NE-stimulated [^{3}H]InsP accumulation. The α_{1b} receptor subpopulation was inactivated by pretreatment of cultures with 100 μM CEC for 30 min. In glial cells, CEC pretreatment caused a highly significant ($p < 0.001$)

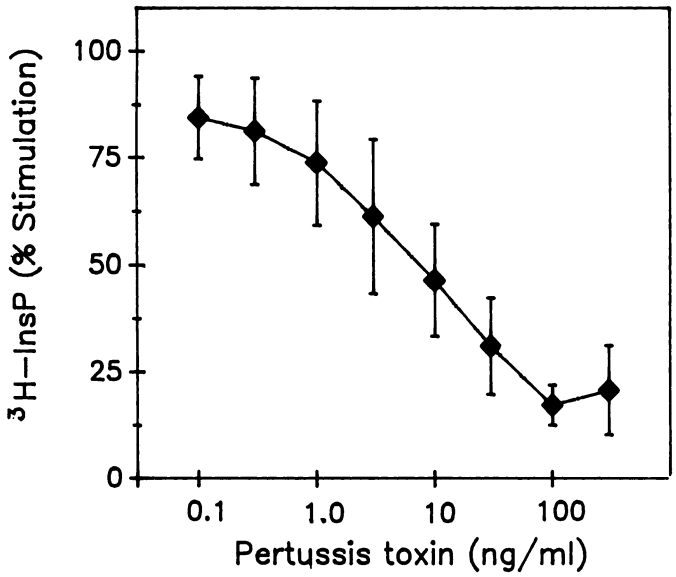

Fig. 2. Inhibition by PTX of NE-stimulated phosphoinositide hydrolysis in glial cultures. Cells were pretreated with various doses of PTX for 15–20 hr, washed, and then incubated with 100 μM NE for 1 hr. Values are expressed as a percentage of NE-stimulated [^{3}H]InsP formation after PTX pretreatment, using the value obtained with 100 μM NE in untreated cells as 100% stimulation. Each *point* represents the mean ± standard error of duplicate determinations from three experiments.

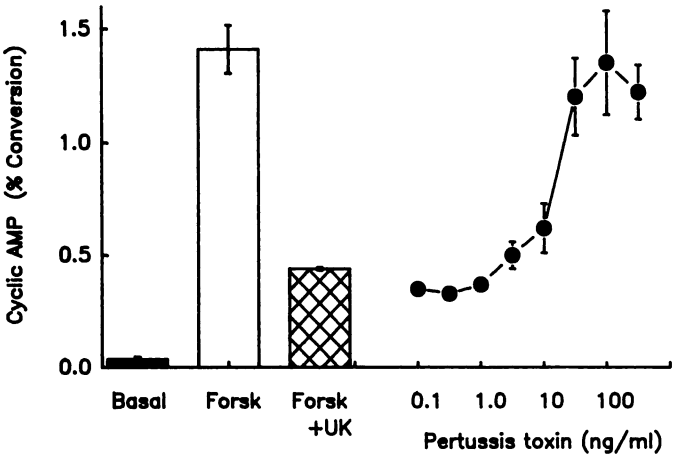

Fig. 3. Effect of PTX on α_2 receptor-mediated inhibition of forskolin-stimulated cAMP accumulation in glial cultures. Cells were pretreated with various doses of PTX for 15–20 hr, pretreated with [^{3}H]adenine for 2 hr, washed, and incubated with either no drug (■), 0.5 μM forskolin (□), or forskolin plus 10 μM UK 14,304 (▩) (*PTX-treated cells*) for 10 min. cAMP accumulation was measured as described in Experimental Procedures. Each *point* represents the mean ± standard error of triplicate determinations from a single experiment, typical of three similar experiments.

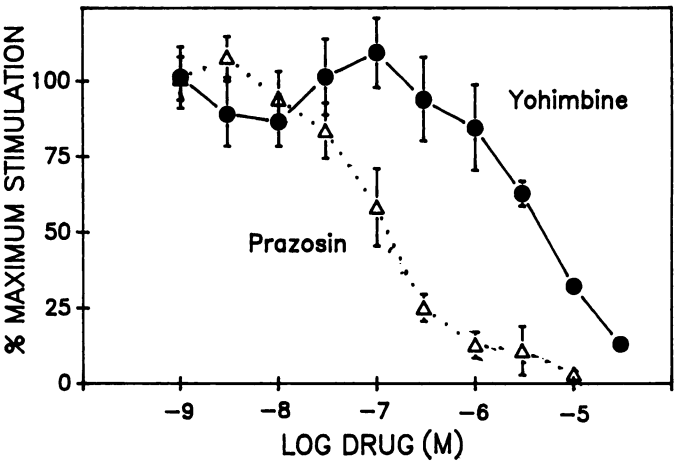

Fig. 4. Inhibition of NE-stimulated [^{3}H]InsP formation by prazosin and yohimbine in glial cells. The concentration of NE was 100 μM. Each *point* is the mean ± standard error of triplicate determinations from three experiments.

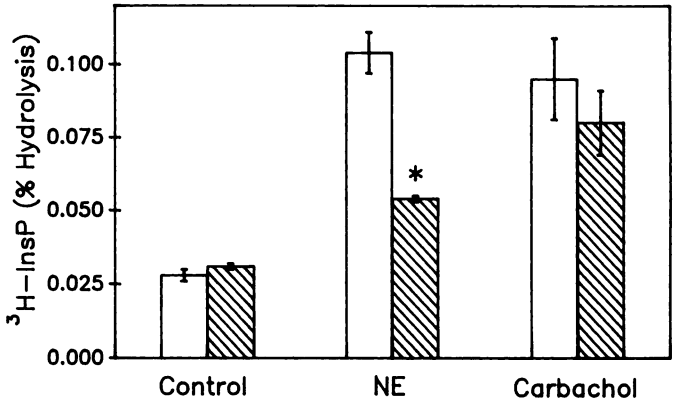

Fig. 5. Effect of PTX on the [^{3}H]InsP response to NE and carbachol in glial cultures. Cells were pretreated with 300 ng/ml PTX for 15–20 hr, washed, and then incubated with either no drug, 100 μM NE, or 1 mM carbachol for 1 hr. □, Control; ■, PTX-pretreated cells. Values represent the mean ± standard error of triplicate determinations from three experiments. *, $p < 0.01$.

TABLE 1

Effect of CEC pretreatment on ^{125}I-BE binding sites in primary neuronal and glial cultures

Cells were scraped off dishes, resuspended in PBS, and homogenized. Following centrifugation, the pellet was resuspended in PBS and incubated for 10 min in the presence or absence of 100 μM CEC at 37°. Membranes were then washed and resuspended in PBS for ^{125}I-BE binding. Each value represents the composite obtained from three or four separate Scatchard analyses.

	B_{max}		K_D	
	Control	CEC	Control	CEC
	fmol/mg of protein		*pM*	
Neurons	181	75	20	41
Glia	73	25	40	81

increase in the EC_{50} of NE, from 1.3 to 11.6 μM, without changing the maximum response (Fig. 6). In neurons, CEC pretreatment caused a smaller but still significant ($p < 0.001$) increase in the EC_{50} of NE, from 0.9 to 2.8 μM, but caused a flattening of the dose-response curve. Concentration-response curves for the effect of CEC pretreatment on responses to submaximal (3 μM) and maximal (300 μM) concentrations of NE were examined in glial cultures (Fig. 7). CEC at 100 μM was found to be maximal for both concentrations of NE.

Combined treatment of CEC and PTX. Pretreatment of glial cells with CEC reduced the [^{3}H]InsP response to 100 μM NE by 25 ± 5%, whereas pretreatment with PTX reduced it by 75 ± 2%. Pretreatment with both PTX and CEC caused a larger reduction than either treatment alone (90 ± 3%; $p < 0.025$ compared with PTX alone; Fig. 8). Similar results were obtained in neurons; CEC reduced the response by 30 ± 5%, PTX by 70 ± 12%, and CEC plus PTX by 91 ± 4% (three experiments; data not shown). In CEC-pretreated cells, the IC_{50} of PTX for inhibiting the [^{3}H]InsP response to NE was 4 ±

TABLE 2

Inhibition of ^{125}I-BE binding by WB 4101 in neuron and glial cell cultures

Inhibition of specific ^{125}I-BE binding was determined and analyzed as described in Experimental Procedures. For one-site analysis, inhibition curves for each cell type were averaged and analyzed by nonlinear regression. For two-site analysis, individual curves were analyzed by nonlinear regression to determine affinities and proportions for the best two-site fit, which were then averaged. Curves in both neurons and glial cells were significantly ($p < 0.05$) better fit by a two-site model (partial F test > 4.1 at 2 and 10 degrees of freedom). Each value is the mean or mean ± standard error of data from four to six experiments.

	Neurons	Glia
One-site analysis		
K_D (nM)	5.0	2.7
n_H	0.74	0.71
Two-site analysis		
K_{D_1} (nM)	1.5 ± 0.25	0.5 ± 0.19
K_{D_2} (nM)	25.1 ± 12.7	8.9 ± 3.20
R_H (%)	47 ± 4.9	35 ± 12.7
R_L (%)	52 ± 4.9	64 ± 12.7

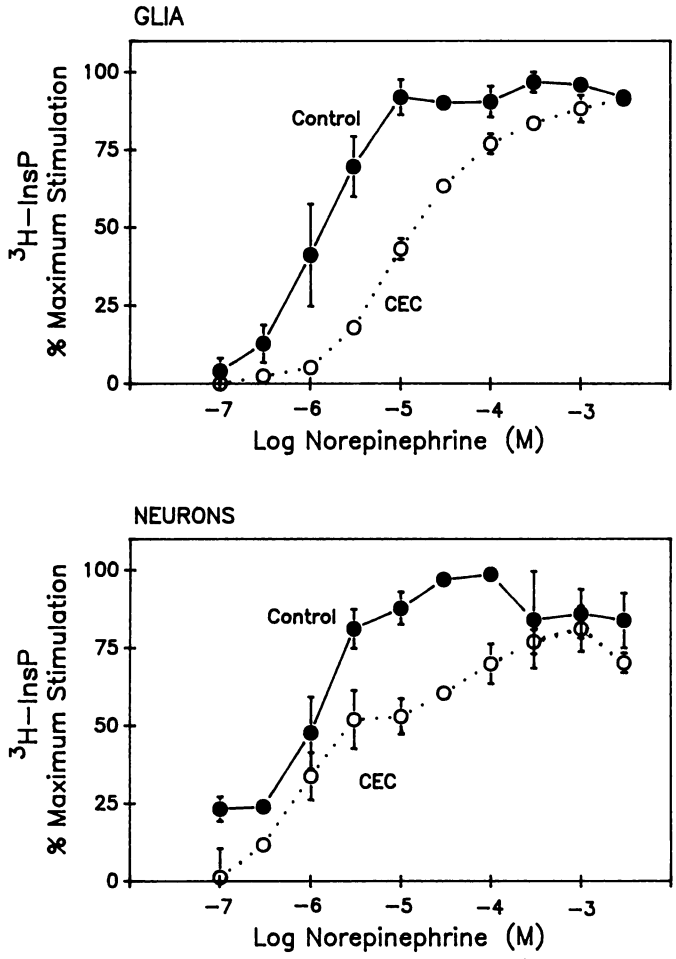

Fig. 6. Effect of CEC pretreatment on NE-stimulated [^{3}H]InsP accumulation in glial cells and neurons. Cultures were pretreated with (○) or without (●) 100 μM CEC for 30 min and washed, and dose-response curves to NE were determined as described in text. Each *point* is the mean ± standard error of duplicate determinations from three experiments.

1.2 ng/ml (three experiments; data not shown), similar to that in control cells.

Role of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} caused a 64 ± 6% decrease in NE-stimulated [^{3}H]InsP accumulation (Fig. 9). In CEC-pretreated cells, removal of extracellular Ca^{2+} caused a greater decrease in the NE response than was caused by either treatment alone.

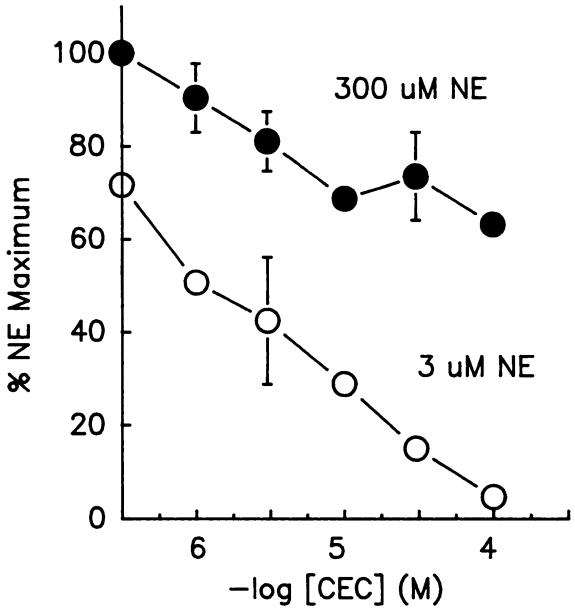

Fig. 7. Concentration-response curve for CEC inhibition of [^{3}H]InsP responses to submaximal (3 μM) and maximal (300 μM) concentrations of NE. Cultures were pretreated for 30 min with the indicated concentrations of CEC and washed three times. [^{3}H]InsP formation in response to 3 μM (○) or 300 μM (●) NE was then determined. Each value is the mean ± standard error of duplicate determinations from two experiments.

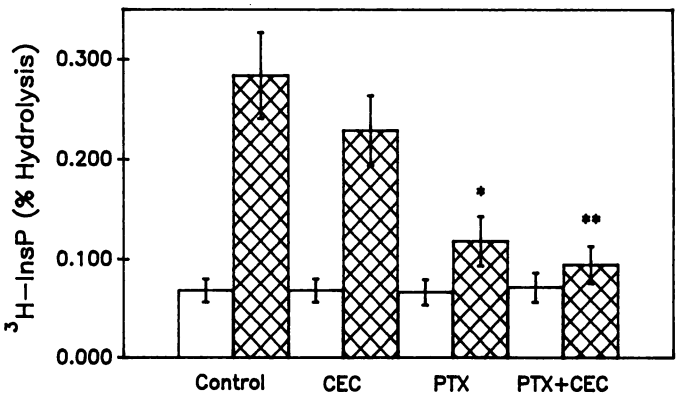

Fig. 8. Inhibition of NE-stimulated [^{3}H]InsP formation by CEC, PTX, or both in glial cells. Half of the cells were treated with 300 ng/ml PTX for 15–20 hr. All cells were washed, and half of the controls and half of PTX-treated cells were incubated for 30 min with 100 μM CEC. All cells were then washed and incubated in the presence or absence of 100 μM NE for 1 hr. Basal levels were not different between groups. Values are the mean ± standard error of triplicate determinations from three experiments. *, $p < 0.01$; **, $p < 0.001$, compared with control.

To determine whether Ca^{2+} influx was necessary for the [^{3}H]InsP response to NE, the effects of organic and inorganic calcium entry blockers were tested. Treatment with 1 μM nifedipine had no effect on the [^{3}H]InsP response to 100 μM NE, whereas 1 mM cadmium chloride decreased it by 56 ± 13% (Fig. 10). Nifedipine had no effect from 0.001 to 10 μM (data not shown). Cadmium caused no inhibition at 100 μM and began to precipitate in the KRB at concentrations higher than 1 mM (not shown).

Discussion

Pretreatment with PTX markedly inhibited NE-stimulated [^{3}H]InsP formation in cultured brain cells. NE appears to be

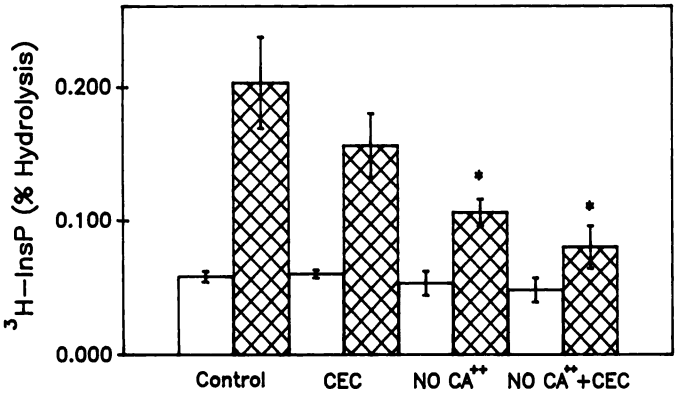

Fig. 9. Inhibition of NE-stimulated [^{3}H]InsP formation by removal of Ca^{2+}, CEC pretreatment, or both in glial cell cultures. Half of the cells were treated with 100 μM CEC for 30 min. All cells were washed three times with either Li-KRB or Ca^{2+}-free Li-KRB and then incubated in the presence ⊠ or absence □ of 100 μM NE with or without Ca^{2+}. Basal levels were not different between groups. Values are the mean ± standard error of triplicate determinations from three experiments. *, $p < 0.05$, compared with control.

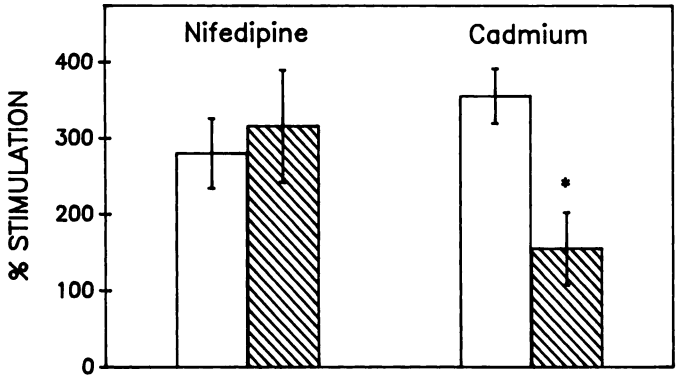

Fig. 10. Effect of Ca^{2+} channel blockers on NE-stimulated [^{3}H]InsP formation in glial cells. Cells were washed and incubated with either 1 μM nifedipine or 1 mM $CdCl_2$, in the presence or absence of 100 μM NE, for 1 hr. Values represent the mean ± standard error of triplicate determinations of the percentage of NE stimulation in the presence (▨) or absence (□) of antagonists from three experiments.

acting through α_1-adrenergic receptors, because prazosin was 40 times more potent than yohimbine in blocking this response. This suggests that α_1-adrenergic receptors activate [^{3}H]InsP formation primarily through a PTX-sensitive G protein in these cells.

Similar inhibition by PTX was observed in both neuronal and glial cultures, although the response to NE was substantially lower in neuronal cultures. There is always some cross-contamination of neuronal cultures with glial cells (5–20%), which could account for the fact that the results were qualitatively similar in the two cell types. However, we have found that the response per cell is much lower in glial cells than in neurons,[3] although the higher density of glial cells causes a much larger total response. This makes it unlikely that the response in neuronal cultures is due primarily to glial contamination.

The potency of PTX in blocking the α_1 response was similar to that for reversing α_2 receptor-mediated decreases in cAMP accumulation. The inhibitory effect of PTX on α_2-adrenergic receptors is caused by ADP-ribosylation of the G_i protein, which inhibits adenylate cyclase (43). It is possible that the G protein mediating α_1-stimulated [^{3}H]InsP formation is the same as that mediating α_2 inhibition of adenylate cyclase. However, there are at least four G protein α subunits, which have similar sensitivities to PTX (α_o, α_{i1}, α_{i2}, and α_{i3}) (13). G_o and G_i have both been identified in neuronal and glial cell cultures (44), and it is not clear which PTX substrate is involved in this response. Although we do not have direct information on the G proteins present in these cultures, the marked inhibition of adenylate cyclase by α_2 receptor activation indicates the presence of at least one functional G_i protein.

The effect of PTX appeared to be specific to the α_1 response in these cells, because PTX pretreatment caused only a small inhibition of the InsP response to carbachol, which was not statistically significant. InsP responses to carbachol have been found to be blocked by PTX in some cells (10) but not others (14, 15). Pearce and Murphy (45) recently showed that the InsP response to carbachol was additive with the response to NE in cultured astrocytes and suggested that different coupling mechanisms or lipid pools were used by the two receptor types. Our data suggest that these receptors may control phospholipase C through different G proteins in these cells. An equally viable explanation would be that these receptors are located on different cell populations. At present we have no data for or against this possibility.

Inhibition of α_1 receptor-stimulated InsP formation by PTX is consistent with earlier work by Moreno *et al.* (21), who showed that PTX reduced NE-stimulated incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol in rat adipocytes. In addition, one other study found a partial (40–50%) inhibition of NE-stimulated InsP formation by PTX in cultured rat cardiomyocytes (46). However, most other studies have found that PTX pretreatment does not alter α_1 receptor-stimulated InsP formation. These include studies in rat cardiomyocytes (22), rat hepatocytes (23), hamster brown adipocytes (24), FRTL-5 rat thyroid cells (25), Madin Darby canine kidney cells (26), hamster BC3H1 smooth muscle cells (26), and rat liver plasma membranes (16).

PTX pretreatment does, however, inhibit certain other α_1-mediated responses. These include negative chronotropic responses in cultured cardiomyocytes (27), activation of arachidonic acid release and stimulation of iodide efflux in FRTL-5 thyroid cells (25, 30), and pressor responses dependent on extracellular Ca^{2+} in pithed rats (31). Other α_1-mediated responses, including positive chronotropic responses in cultured cardiomyocytes (27), positive inotropic responses in rat ventricle (28), and activation of cyclic AMP degradation in rat cardiomyocytes (29), have not been found to be affected by PTX pretreatment.

The differential effects of PTX on α_1-stimulated responses may be related to the recently identified subtypes of α_1-adrenergic receptors. Two pharmacologically distinct subtypes have been identified using radioligand binding and functional assays (32–35). The α_{1b} subtype is sensitive to inactivation by CEC and has a low affinity for the competitive antagonist WB 4101, whereas the α_{1a} subtype has a higher affinity for WB 4101 and is insensitive to CEC inactivation (34, 35). Radioligand binding data presented here suggest that both neuronal and glial cell cultures contain mixed populations of both subtypes.

The α_{1b} subpopulation can be inactivated by pretreatment with CEC (34, 37). This treatment caused little or no reduction in the maximal [^{3}H]InsP response to NE in these cultures, suggesting that activation of the α_{1a} subtype causes substantial

increases in [^{3}H]InsP formation. This is in agreement with our recent evidence in collagenase-dispersed hepatocytes and renal cells, showing that both α_{1a} and α_{1b} receptor subtypes can activate InsP formation[1]. The proportion of this response inactivated by CEC was essentially additive with that inactivated by PTX, although it is difficult to distinguish such additivity from a similar proportional effect before and after CEC pretreatment. These results raise the possibility that α_{1b}-mediated [^{3}H]InsP formation was independent of PTX inhibition and that the PTX-sensitive G protein may couple selectively with the α_{1a} subtype.

Han *et al.* (33) observed that α_{1a}-mediated contractions of smooth muscle required extracellular Ca^{2+}, whereas α_{1b}-mediated contractions were independent of extracellular Ca^{2+}. They proposed that α_{1a} receptors might control influx of extracellular Ca^{2+}, whereas α_{1b} receptors controlled release of stored intracellular Ca^{2+} by formation of Ins(1,4,5)P_3. This correlation has been supported by recent work of Tsujimoto *et al.* (47) and Hanft and Gross (48). We have recently found, however, that both subtypes will activate [^{3}H]InsP formation[1], although the types of InsPs formed and the mechanisms[1] involved have yet to be determined.

Ca^{2+} requirements for receptor-mediated inositol lipid metabolism are variable, but most agonist-stimulated [^{3}H]InsP formation is only partially dependent on Ca^{2+}. In astrocytes, the absence of Ca^{2+} reduced [^{3}H]InsP formation elicited by NE, carbachol, and glutamate; addition of EGTA abolished the response (45). In the present study, influx of extracellular Ca^{2+} appeared to be necessary for a large proportion of NE-stimulated InsP accumulation. It has been suggested that differences in Ca^{2+} sensitivities may be indicative of different substrate requirements for phospholipase C (49, 50). Both phosphatidylinositol and polyphosphoinositides (phosphatidylinositol monophosphate, PIP_2) have been shown to be substrates for phospholipase C; however, in the absence of Ca^{2+}, PIP_2 (and sometimes phosphatidylinositol monophosphate) is preferentially hydrolyzed, and higher concentrations of Ca^{2+} are required for hydrolysis of phosphatidylinositol (50). Thus, the possibility exists that activation of different α_1 subtypes may preferentially cause hydrolysis of PIP_2 (α_{1b}) or other inositol phospholipids (α_{1a}).

The present results suggest that stimulation of InsP formation by the α_{1a} subtype is subsequent to influx of extracellular Ca^{2+}. We found that removal of extracellular Ca^{2+} or addition of cadmium caused a substantial inhibition of NE-stimulated InsP accumulation. This appeared to be additive with the inhibition caused by CEC, suggesting that the InsP response to the α_{1a} subtype is dependent on extracellular Ca^{2+}, whereas the α_{1b} response is not.

A primary effect of the α_{1a} subtype on Ca^{2+} influx, which is blocked by PTX, would be consistent with functional data in the pithed rat. Nichols *et al.* (31) recently showed that PTX pretreatment blocks α_1-adrenergic receptor-mediated pressor responses, which are dependent on Ca^{2+} influx. Although α_1-mediated Ca^{2+} influx occurs through dihydropyridine-sensitive Ca^{2+} channels in smooth muscle, our data suggest that, in brain cells, Ca^{2+} enters through nifedipine-insensitive channels. Similarly, in rat cortical slices, cobalt selectively reduced NE-stimulated [^{3}H]InsP formation, whereas nifedipine had no effect (51).

These results are in agreement with experiments in hepatocytes showing that epidermal growth factor and vasopressin stimulate Ca^{2+} influx through a PTX-sensitive G protein (52). However, most other studies find that PTX-sensitive G proteins (particularly G_o) inhibit Ca^{2+} influx (53). It should be noted, however, that most receptors inhibiting Ca^{2+} influx through a PTX-sensitive G protein have not been associated with InsP formation. It will be interesting to discover whether different PTX-sensitive G proteins can increase or decrease Ca^{2+} influx, possibly through different channels.

These results support the hypothesis that α_{1a} and α_{1b} receptor subtypes couple to InsP formation through different mechanisms. Each subtype appears to activate a different G protein, distinguished by their sensitivity to PTX. The α_{1b} subtype appears to activate PIP_2 hydrolysis through a PTX-insensitive G protein (G_p), whereas the α_{1a} subtype may promote Ca^{2+} influx through a PTX-sensitive G protein. Ashkenazi *et al.* (54) recently showed that recombinant muscarinic receptor subtypes transfected into the same cells coupled selectively to different G proteins for stimulating InsP formation. These G proteins could also be distinguished by their sensitivity to PTX. This supports the hypothesis that different, but closely related, receptor subtypes act through distinct G proteins. It will be interesting to further characterize such systems and identify the similarities and differences in the molecular events activated by distinct receptor subtypes.

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Send reprint requests to: Kenneth P. Minneman, Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.