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Opioid μ, δ, and κ Receptor-Induced Activation of Phospholipase C-β3 and Inhibition of Adenylyl Cyclase Is Mediated by G_{i2} and G_o in Smooth Muscle

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SUMMARY

In neurons and transformed cell lines, opioid receptors are coupled to various signaling mechanisms involved in Ca^{2+} mobilization, including inhibition or activation of Ca^{2+} channels and phospholipase C-β (PLC-β), the enzyme responsible for generation of the Ca^{2+} mobilizing messenger inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. In the current study, we used selective PLC-β and G protein antibodies to identify the PLC-β isozyme activated by opioid receptors in intestinal smooth muscle and the G proteins to which the PLC-β isozyme and adenylyl cyclase are coupled. [D-Pen^2,D-Pen^5]Enkephalin, a δ receptor agonist, stimulated Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction; inhibited forskolin-stimulated cAMP formation in dispersed muscle cells; and stimulated phosphoinositide hydrolysis in plasma membranes; all of the effects were blocked by pertussis toxin. [D-Pen^2,D-Pen^5]Enkephalin-stimulated Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction in permeabilized muscle cells and phosphoinositide hydrolysis in plasma membranes were selectively blocked by G_β antibody and PLC-β3 antibody; contractions stimulated by [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin, a μ receptor agonist, and U-69,593, a κ receptor agonist, were also blocked by G_β and PLC-β3 antibodies. Inhibition of forskolin-stimulated cAMP formation by δ, μ, and κ receptor agonists was partially blocked by $G_{\alpha i2}$ and $G_{\alpha o}$ antibodies and additively blocked by a combination of the antibodies. The δ, μ, and κ receptor agonists stimulated the binding of guanosine-5′-*O*-(3-thio)triphosphate to the α subunits of G_{i2} and G_o but not to the α subunits of other G proteins. We conclude that opioid μ, δ, and κ receptors are selectively coupled to G_{i2} and G_o in intestinal smooth muscle. The βγ subunits of both G proteins activate PLC-β3, thereby stimulating Ins(1,4,5)P_3-dependent Ca^{2+} release and smooth muscle contraction, whereas the α subunits inhibit adenylyl cyclase activity.

Three opioid receptors, designated μ, δ, and κ, have been cloned from various species, including rat, mouse, and human, and characterized functionally and by radioligand binding (1–6). The receptors exhibit ~60% homology with each other and lesser homology (31–37%) with somatostatin and angiotensin receptors, and they differ in their affinity for endogenous and exogenous opioid ligands (1, 3, 7). The receptors are differentially expressed in the central and peripheral nervous system but are absent from many organs, including the heart, lung, liver, spleen, and kidney (3). In the gastrointestinal tract, opioid receptors are present on enteric neurons and on smooth muscle cells of the circular muscle layer but are absent from smooth muscle cells of the longitudinal muscle layer (8–11).

Three properties common to all opioid receptors have been well characterized: activation of K^+ channels of the inward rectifier family (12–14), inhibition of high voltage-dependent Ca^{2+} channels (N-type channels) (15), and inhibition of adenylyl cyclase activity (1, 3, 16). All three properties are mediated by PTX-sensitive G proteins (1, 14, 16). The coupling of G proteins to activation of K^+ channels and inhibition of Ca^{2+} channels seems to be direct and does not require the participation of a diffusible messenger because it can be maintained in excised membrane patches (3, 13).

Different G proteins mediate inhibition of adenylyl cyclase in various cell lines and neural membranes (16–18). In neuroblastoma glioma hybrid NG108-15 cells, δ receptors are abundantly expressed and are coupled to inhibition of adenylyl cyclase via G_{i2} (16, 19). In human neuroblastoma SH-SY5Y cells, in which μ receptors are more abundantly expressed than δ receptors, μ receptors are coupled to inhibition of adenylyl cyclase, mainly via G_{i3}, whereas δ receptors are coupled mainly via G_{i1}; both receptor types are also coupled to adenylyl cyclase via G_o, although to a lesser extent (17, 20–22).

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ABBREVIATIONS: PTX, pertussis toxin; PLC, phospholipase C; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; PI, phosphoinositide; HEPES, 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DAMGO, [D-Ala^2, *N*-MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin.

More recent studies have shown that in some cell lines, opioid receptors mediate Ca^{2+} mobilization (22, 23). In NG108-15 cells and neuroblastoma X dorsal root ganglion hybrid ND8-47 cells, low concentrations of δ receptor agonists activate dihydropyridine-sensitive Ca^{2+} channels, causing Ca^{2+} influx into the cells, whereas higher concentrations induce additional Ca^{2+} release (23, 24). Ca^{2+} mobilization in both cell lines is sensitive to PTX (23–25). In SH-SY5Y cells, μ receptor agonists stimulate PI hydrolysis, an effect that is mediated by Ca^{2+} influx and abolished by Ca^{2+} channel blockers (22). Although sensitive to PTX, the stimulation of PI hydrolysis in SH-SY5Y cells seems to be an indirect consequence of a G protein-coupled process involving Ca^{2+} influx (21, 22). In other cell lines (e.g., Chinese hamster ovary and COS cells), however, μ receptors mediate inhibition of PLC-β activity via an unidentified inhibitory G protein (26), whereas in synaptosomal cerebellar membranes, κ receptors mediate inhibition of PLC-β activity via G_{i1} (27).

The three opioid receptor types coexist on gastric and intestinal smooth muscle cells of the circular layer in various species (e.g., human, guinea pig, rabbit), where they mediate contraction (8–11). The muscle layer is innervated by enteric dynorphin- and [methionine]enkephalin-immunoreactive nerve fibers (28). Preliminary studies have suggested that contraction was not mediated by Ca^{2+} influx and was sensitive to PTX, which is consistent with involvement of an inhibitory G protein. In the current study, we examined the signaling cascade initiated by opioid receptors in intact and permeabilized intestinal muscle cells and in plasma membranes isolated from these cells. Specific G protein and PLC-β antibodies were used to identify the G protein subunit or subunits and PLC-β isozyme or isozymes mediating PI hydrolysis, Ca^{2+} mobilization, and contraction by opioid agonists and the G protein subunit or subunits mediating inhibition of adenylyl cyclase activity.

Experimental Procedures

Preparation of dispersed intestinal muscle cells. Muscle cells were isolated separately from the circular and longitudinal muscle layers of guinea pig intestine by 30-min incubation at 31° in HEPES medium with Type II collagenase (0.1%) and soybean trypsin inhibitor (0.1%) as described previously (8–11, 29). The muscle strips were washed with 50 ml of enzyme-free medium, and muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-μm Nitex Filters (Tetco, Briarcliff Manor, NY) and centrifuged twice at 350 × *g* for 10 min.

In experiments using G protein or PLC-β antibodies, the cells were permeabilized as described previously (29, 30) through incubation for 10 min with 35 μg/ml saponin in a medium containing 20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.34 mM $CaCl_2$, 1 mM EGTA, and 1% bovine serum albumin. The cells were centrifuged at 350 × *g* for 5 min, washed free of saponin, and resuspended in the same medium with 1.5 mM ATP and ATP-regenerating system (5 mM creatine phosphate and 10 units/ml creatine phosphokinase).

Assay for cAMP in dispersed muscle cells. cAMP was measured in intact and permeabilized circular muscle cells by radioimmunoassay as described previously (29). Cell aliquots (0.5 ml; 10^6 cells/ml) were incubated with 1 μM 3-isobutyl-1-methylxanthine and various concentrations of DPDPE, 1 μM DAMGO, or 1 μM U-69,593 {5α,7α,8β-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl]benzeneacetamide}, and the reaction was terminated after 60 sec with 6% cold trichloroacetic acid (v/v). The mixture was centrifuged at 2000 × *g* for 15 min at 4°, and the supernatant was extracted with diethyl ether and lyophilized. The samples were reconstituted for radioimmunoassay in 500 μl of 50 mM sodium acetate, pH 6.2, and acetylated with triethylamine/acetic anhydride (3:1 v/v) for 30 min. cAMP was measured in duplicate using 100-μl aliquots and expressed as pmol/10^6 cells.

Radioreceptor assay for Ins(1,4,5)P_3 in dispersed muscle cells. Ins(1,4,5)P_3 was measured in intact and permeabilized circular muscle cells as described previously (29, 30) using an assay system (Amersham, Arlington Heights, IL) that uses ^{3}H-labeled D-*myo*-Ins(1,4,5)P_3 and bovine brain microsomes. One milliliter of muscle cell suspension (10^6 cells/ml) was incubated with 10 mM Li^+ for 10 min at 31°, after which DPDPE was added for 30 sec, and the reaction terminated with ice-cold 10% perchloric acid. After the mixture was centrifuged for 10 min at 750 × *g*, the supernatant was extracted, and Ins(1,4,5)P_3 content in the aqueous phase was measured. Results were computed from a standard curve and expressed as pmol/10^6 cells.

Measurement of Ca^{2+} release in dispersed muscle cells. Ca^{2+} release was measured in intact and permeabilized muscle cells by an adaptation of the method of Poggioli and Putney (31) as described previously (29, 30). The cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml), and Ca^{2+} uptake was measured at intervals for 90 min when a steady state was attained. DPDPE was then added, and $^{45}Ca^{2+}$ cell content was measured after 30 sec. Ca^{2+} release was expressed as percentage decrease in steady state $^{45}Ca^{2+}$ cell content. In other experiments, G protein or PLC-β antibodies were added 30 min after ATP, and the incubation was maintained for an additional 60 min. The addition of antibodies had no effect on steady state Ca^{2+} uptake.

Measurement of muscle cell contraction by scanning micrometry. Contraction was measured in intact and permeabilized muscle cells by scanning micrometry as described previously (8–11, 29, 30). A 0.25-ml aliquot of cell suspension containing 10^4 muscle cells/ml was added to 0.1 ml of medium containing DPDPE, DAMGO, or U-69,593, and the reaction was terminated after 30 sec with 1% acrolein. The effect of PLC-β and G protein antibodies was determined in permeabilized muscle cells after preincubation for 1 hr with 10 μg/ml concentration of each antibody separately. The lengths of muscle cells treated with DPDPE, DAMGO, or U-69,593 were measured and compared with the length of untreated cells. Contraction was expressed as percentage decrease in mean cell length from control. Concentration-response studies showed that the highest response to DPDPE was obtained at a concentration of 1 μM.

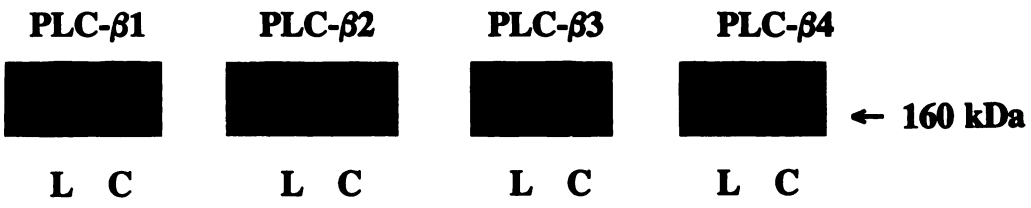

Fig. 1. Western blot analysis of PLC-β isozymes in homogenates of dispersed intestinal circular (*C*) and longitudinal (*L*) muscle cells. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with specific antibodies to PLC-β isozymes and anti-rabbit IgG conjugated to horseradish peroxidase.

Assay of PLC-β activity in plasma membranes. PLC-β activity was determined in plasma membranes prelabeled with *myo*-[^{3}H]inositol by a modification of the method of Uhing *et al.* (32) as described previously (29, 33). A plasma membrane fraction was first obtained and then centrifuged at 200,000 × *g* for 2 hr and resuspended in 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 100 μg/ml leupeptin, and 100 μg/ml antipain, yielding a final concentration of 2 mg of protein/ml (10,000–20,000 cpm/mg of protein); the membranes were used immediately for assay of PLC-β activity. PLC-β assay was initiated by the addition of 0.4 mg of membrane protein to 25 mM Tris·HCl, pH 7.5, 0.5 mM EGTA, 10 mM $MgCl_2$, 300 nM free Ca^{2+}, 10 μM GTP, 5 mM phosphocreatine, and 50 units/ml creatine phosphokinase in a total volume of 0.4 ml. After incubation at 31° for 60 sec, the reaction was terminated with 0.6 ml of 25% trichloroacetic acid (w/v). The supernatant was extracted four times with 2 ml of diethyl ether, and the amount of labeled inositol phosphates in the aqueous phase was counted. The trichloroacetic acid-soluble radioactivity at zero time (~150 cpm) was subtracted from all values. PLC-β activity was expressed as cpm/mg of protein-min.

Identification of PLC-β isozymes by Western blot. Western blot analysis of PLC-β isozymes was determined as described previously (33). Intestinal circular and longitudinal muscle cells (2×10^6 cells/ml) were homogenized in a medium containing 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, and 2 μg/ml leupeptin and centrifuged at 12,000 × *g* for 5 min. The supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred at 4° to nitrocellulose membranes. The blots were incubated for 1 hr at room temperature in Tris-buffered saline containing 150 mM NaCl and 50 mM Tris·HCl, pH 7.5, in 5% nonfat dry milk to block nonspecific antibody binding. After three or four washes in Tris-buffered saline, the blots were incubated for 12 hr at 4° with isozyme-specific PLC-β antibodies (1 μg/ml) and, after further washing, for 1 hr with anti-rabbit IgG conjugated with horseradish peroxidase. The bands were identified by enhanced chemiluminescence reagents.

Identification of receptor-activated G proteins in smooth muscle membranes. G proteins selectively activated by opioid ligands were identified according to the method of Okamoto *et al.* (34). Muscle cells were washed twice in phosphate-buffered saline and homogenized in a medium containing 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM DTT. After centrifugation at 27,000 × *g* for 15 min, the crude membranes were solubilized in a medium containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 240 mM NaCl, and 1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate for 60 min at 4°. The membranes were incubated for various periods at 37° with 60 nM [^{35}S]GTPγS in a solution containing 10 mM HEPES, pH 7.4, 100 μM EDTA, and 10 mM $MgCl_2$. Ten volumes of 100 mM Tris·HCl, pH 8.0, medium containing 10 mM $MgCl_2$, 100 mM NaCl, and 20 μM GTP was added to the membranes, and the mixture was placed into wells precoated with G protein antibodies. After incubation for 2 hr on ice, the solution was discarded, and the wells were washed three times with PBS containing 0.05% Tween-20. The radioactivity from each well was counted by liquid scintillation. Coating with G protein antibodies (1:1000) was done after the wells were first coated with anti-rabbit IgG (1:1000) for 2 hr on ice.

Data analysis. Results were calculated as mean ± standard error of a varying number of experiments using cell suspensions obtained from different animals. Statistical significance was tested using Student's *t* test for paired or unpaired values.

Materials. Monoclonal antibody to PLC-β1 was obtained from Upstate Biotechnology (Lake Placid, NY); polyclonal antibodies to PLC-β2, PLC-β3, PLC-β4, $G_{\alpha o}$, and $G_{\alpha q/11}$ were from Santa Cruz Biochemicals (Santa Cruz, CA); PTX and antibodies to $G_{\alpha i1}$, $G_{\alpha i1\text{-}2}$, $G_{\alpha i3}$, $G_{\alpha s}$, and G_β were from Calbiochem (San Diego, CA); monoclonal antibody to $G_{\alpha i2}$ was from Chemicon International (Temecula, CA); ^{125}I-cAMP, *myo*-[^{3}H]inositol, [^{35}S]GTPγS, and $^{45}CaCl_2$ were from DuPont-New England Nuclear (Boston, MA); nitrocellulose membranes were from BioRad (Richmond, CA); collagenase Type II and soybean trypsin inhibitor were from Worthington Biochemicals (Freehold, NJ); D-*myo*-Ins(1,4,5)P_3 assay system was from Amersham; and DAMGO, DPDPE, and U-69,593 were from Research Biochemicals (Natick, MA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Results

Expression of PLC-β isozymes in intestinal smooth muscle cells. Western blot analysis disclosed the presence of PLC-β1, PLC-β2, PLC-β3, and PLC-β4 in homogenates derived separately from dispersed intestinal circular and longitudinal muscle cells (Fig. 1). Western blot analysis of G proteins disclosed the presence of G_q, G_s, G_{i1}, G_{i2}, G_{i3}, and G_o (35).

Identification of opioid receptor-activated G proteins. The G protein antibodies used in Western blot analysis were used to identify the G protein or proteins activated by the δ receptor agonist DPDPE, the μ receptor agonist DAMGO, and the κ receptor agonist U-69,593. Incubation of muscle cell membranes with each agonist and [^{35}S]GTPγS for 20 min caused a significant, time-dependent increase in the binding of [^{35}S]GTPγ/G_α complexes to wells precoated with antibodies to $G_{\alpha i1\text{-}2}$, $G_{\alpha i2}$, and $G_{\alpha o}$ but not to wells precoated with antibodies to $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha q/11}$, or $G_{\alpha s}$ (Fig. 2 and Table 1). The increase in bound radioactivity reflected selective binding of [^{35}S]GTPγS to the dissociated α subunits of G_{i2} and G_o, with subsequent binding of the GTPγS/G_α complexes to $G_{\alpha i2}$ and $G_{\alpha o}$ antibodies. The pattern of selective activation of G_o and G_{i2} was identical for δ, μ, and κ receptor agonists.

Response of intact intestinal circular muscle cells to δ receptor agonists. DPDPE caused contraction of dispersed intestinal circular muscle cells and stimulated Ca^{2+}

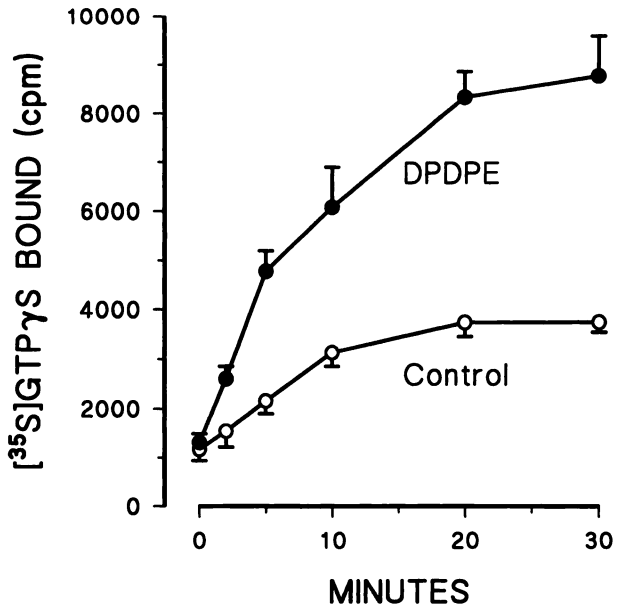

Fig. 2. Binding of DPDPE-stimulated GTPγS/G_α complexes in smooth muscle membranes to $G_{\alpha i1\text{-}2}$ antibody. Membranes isolated from dispersed intestinal circular muscle cells were incubated with [^{35}S]GTPγS in the absence or presence of 1 μM DPDPE for various periods at 37°. Aliquots were added to wells precoated with $G_{\alpha i1\text{-}2}$ antibody for 2 hr, and bound radioactivity was measured. Values are mean ± standard error of five experiments.

TABLE 1

Binding of opioid agonist-stimulated GTPγS-G_α complexes in smooth muscle membranes to specific G protein antibodies

Crude membranes isolated from dispersed intestinal circular muscle cells were incubated with [^{35}S]GTPγS in the absence or presence of 1 μM DPDPE (δ receptor agonist), DAMGO (μ receptor agonist), or U-69,593 (κ receptor agonist) for 20 min at 37°. Aliquots were added to wells precoated with specific G protein antibodies for 2 hr, and bound radioactivity in each well was measured. The nonspecific binding of GTPγS to the antibody-coated wells in the absence of membranes (234 ± 56 cpm) was subtracted. Values are mean ± standard error of four experiments.

Antibody	[^{35}S]GTPγS-G protein complex bound			
	Basal	+DPDPE	+DAMGO	+U-69,593
		cpm		
$G_{\alpha i1}$	3,444 ± 388	3,900 ± 401	4,303 ± 764	4,172 ± 462
$G_{\alpha i2}$	4,010 ± 193	10,070 ± 474[a]	10,061 ± 464[a]	10,142 ± 532[a]
$G_{\alpha i3}$	3,646 ± 377	3,785 ± 625	3,660 ± 486	3,696 ± 426
$G_{\alpha o}$	3,643 ± 377	6,134 ± 666[a]	5,863 ± 818[a]	6,654 ± 566[a]
$G_{\alpha s}$	4,038 ± 393	3,893 ± 403	4,126 ± 499	4,114 ± 498
$G_{\alpha q/11}$	3,544 ± 381	3,988 ± 652	3,517 ± 347	3,852 ± 333

[a] $p < 0.01$.

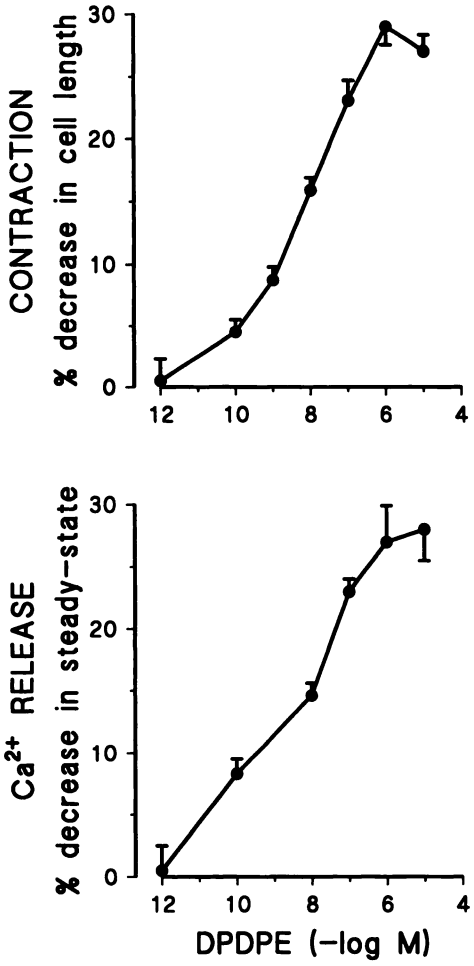

Fig. 3. Concentration-response curves for the effect of DPDPE on muscle contraction and Ca^{2+} release in dispersed intestinal circular muscle cells. Contraction was measured by scanning micrometry and expressed as percentage decrease in cell length from control (116 ± 3 μm). Ca^{2+} release was expressed as percentage decrease in steady state Ca^{2+} content (2.21 ± 0.32 nmol/10^6 cells) in cells preloaded with $^{45}Ca^{2+}$. Values are means ± standard error of four experiments.

release in a concentration-dependent fashion with EC_{50} values of 5 ± 3 and 2 ± 1 nM, respectively (Fig. 3). Maximal contraction (27.5 ± 2.5% decrease in cell length) and Ca^{2+} release (27 ± 3% decrease in steady state $^{45}Ca^{2+}$ cell content) were elicited by 1 μM DPDPE. At the same concentration, DPDPE increased Ins(1,4,5)P_3 formation by 5.4 ± 0.6 pmol/

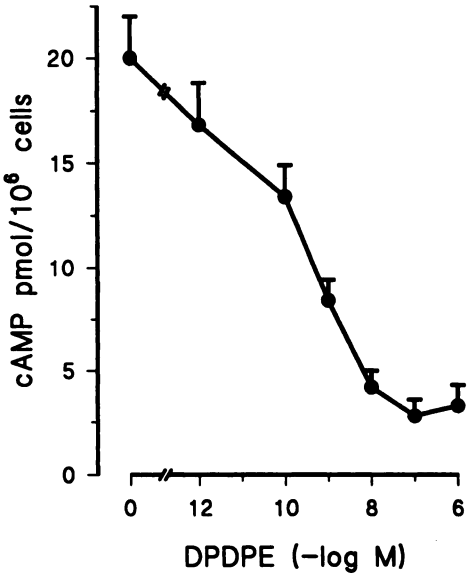

Fig. 4. Concentration-response curve for inhibition of forskolin-stimulated cAMP formation by DPDPE in dispersed intestinal muscle cells. Muscle cells were treated with 10 μM forskolin alone or in the presence of DPDPE for 60 sec. Basal cAMP, 4.2 ± 0.2 pmol/10^6 cells; forskolin-stimulated cAMP, 20.1 ± 2.4 pmol/10^6 cells above basal level. Values are mean ± standard error of three experiments.

10^6 cells above basal levels (basal, 3.5 ± 0.3 pmol/10^6 cells). DPDPE also inhibited forskolin-stimulated cAMP formation in a concentration-dependent fashion, with an EC_{50} value of 0.2 ± 0.1 nM (Fig. 4). Maximal inhibition of cAMP was obtained with 0.1 μM DPDPE (forskolin alone, 20.1 ± 2.1 pmol/10^6 cells; forskolin plus DPDPE, 2.8 ± 0.4 pmol/10^6 cells, or 86 ± 2% inhibition).

Treatment of the cells for 60 min with PTX (800 ng/ml) inhibited maximal DPDPE-induced Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction by 78 ± 3%, 79 ± 5%, and 85 ± 8%, respectively (Table 2). Treatment with PTX also reversed the inhibition of forskolin-stimulated cAMP formation (forskolin, 20.6 ± 1.2 pmol/10^6 cells; forskolin plus DPDPE, 4.6 ± 0.5 pmol/10^6 cells; forskolin plus DPDPE and PTX, 16.3 ± 1.3 pmol/10^6 cells) (Table 2).

Identification of PLC-β isozymes and G proteins functionally coupled to δ receptors. PI-specific PLC-β activity was measured in plasma membranes derived from dispersed circular muscle cells. PLC-β activity (inositol phosphate formation) in membranes increased by 454 ± 49% on

TABLE 2

Effect of PTX on DPDPE-induced PLC-β activity in plasma membranes and Ins(1,4,5)P_3 formation, Ca^{2+} release, muscle contraction, and forskolin-stimulated cAMP in dispersed intestinal muscle cells

PLC-β activity was expressed as increased inositol phosphate formation above basal levels (608 ± 75 cpm/mg protein-min). Ins(1,4,5)P_3 mass was measured by radioreceptor assay and expressed as an increase in pmol/10^6 cells above basal levels (3.50 ± 0.3 pmol/10^6 cells). Ca^{2+} release was expressed as percentage decrease in steady state Ca^{2+} content (2.42 ± 0.36 nmol/10^6 cells) in cells preloaded with $^{45}Ca^{2+}$. Contraction was measured by scanning micrometry and expressed as percentage decrease in control cell length (116 ± 3 μm). cAMP was measured in the presence of 10 μM forskolin, and the results are expressed as pmol/10^6 cells above basal levels (basal, 4.5 ± 0.3 pmol/10^6 cells; increase with forskolin, 20.6 ± 1.2 pmol/10^6 cells). Values are mean ± standard error of four or five experiments.

	DPDPE (1 μM)	DPDPE + PTX	Inhibition
			%
PLC-β (cpm/mg-min)	2067 ± 224	288 ± 53[a]	86 ± 4
Ins(1,4,5)P_3 (pmol/10^6 cells)	5.4 ± 0.6	1.2 ± 0.2[a]	78 ± 3
Ca^{2+} release (Δ%)	29.2 ± 3.3	6.2 ± 1.6[a]	79 ± 5
Contraction (Δ%)	27.5 ± 2.5	4.3 ± 2.4[a]	85 ± 8
cAMP (pmol/10^6 cells)	4.6 ± 0.5[a]	16.3 ± 1.3	19 ± 3

[a] $p < 0.01$.

the addition of 10 μM GTP and 1 μM DPDPE. Pretreatment of the cells for 1 hr with 800 ng/ml PTX before membrane isolation inhibited PLC-β activity in plasma membranes by 86 ± 4% (Table 2). Pretreatment of plasma membranes for 1 hr with 10 μg/ml PLC-β3 antibody inhibited PLC-β activity by 68 ± 2% (Table 3), whereas pretreatment with 10 μg/ml PLC-β1, PLC-β2, or PLC-β4 antibody had no significant effect (range of inhibition, 1 ± 7% to 5 ± 8%; p = NS).

Pretreatment of plasma membranes with 10 μg/ml common G_β antibody inhibited DPDPE-stimulated PLC-β activity by 67 ± 4%, whereas pretreatment with 10 μg/ml $G_{\alpha i1\text{-}2}$, $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha o}$, $G_{\alpha s}$, or $G_{\alpha q/11}$ antibody had no significant effect (range of inhibition, 1 ± 2% to 4 ± 9%; p = NS) (Table 3).

The effect of PLC-β and G protein antibodies on Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction was examined in permeabilized muscle cells. Permeabilization had no effect on DPDPE-induced contraction (27.5 ± 2.5% decrease in cell length in intact cells versus 26.8 ± 1.8% in permeabilized cells) (Tables 2 and 3). Pretreatment of the cells with 10 μg/ml G_β antibody inhibited DPDPE-induced Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction by 68 ± 7%, 83 ± 3%, and 84 ± 5%, respectively (Table 3). Pretreatment of the cells with 10 μg/ml PLC-β3 antibody had a similar effect, inhibiting DPDPE-induced Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction by 80 ± 3%, 84 ± 5%, and 84 ± 3%, respectively (Table 3). No other G protein or PLC-β antibody had a significant effect on Ins(1,4,5)P_3 formation, Ca^{2+} release, or contraction (range of inhibition, 2 ± 8% to 10 ± 9%; p = NS) (Table 3). Contraction and Ca^{2+} release induced by exogenous Ins(1,4,5)P_3 (1 μM) were not affected by pretreatment of the cells with either G_β or PLC-β3 antibody (inhibition, 2 ± 7% and 1 ± 7%; p = NS).

Identification of G proteins coupled to inhibition of adenylyl cyclase by δ receptors. G protein antibodies were also used to explore the coupling of δ receptors to inhibition of adenylyl cyclase in permeabilized muscle cells. Forskolin stimulated cAMP formation to the same extent in intact (20.1 ± 2.4 pmol/10^6 cells) and permeabilized (21.4 ± 2.0 pmol/10^6 cells) muscle cells, and DPDPE (1 μM) inhibited forskolin-stimulated cAMP formation to the same extent in intact (78 ± 4%) and permeabilized (77 ± 3%) muscle cells. Pretreatment of permeabilized muscle cells with 10 μg/ml $G_{\alpha i1\text{-}2}$, $G_{\alpha i2}$, or $G_{\alpha o}$ antibody partly reversed DPDPE-induced inhibition of forskolin-stimulated cAMP to 48 ± 2%, 56 ± 8%, and 52 ± 8%, respectively (Fig. 5). Pretreatment with 10 μg/ml $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha q/11}$, or G_β had no significant effect on DPDPE-induced inhibition of forskolin-stimulated cAMP formation (control, 77 ± 3% inhibition of cAMP; range with G protein antibodies, 73 ± 3% to 77 ± 6%).

The effect of 10 μg/ml $G_{\alpha i2}$ or $G_{\alpha o}$ antibody was optimal because it was not exceeded when a higher concentration (20 μg/ml) was used (45 ± 3% and 47 ± 2% inhibition). The effect of a combination of $G_{\alpha i2}$ and $G_{\alpha o}$ antibodies, however, was additive, reversing inhibition to 20 ± 4% when a 10 μg/ml concentration of each antibody was used, and 18 ± 5% when a 20 μg/ml concentration of each antibody was used (Fig. 5). A combination of G_β antibody with $G_{\alpha i2}$ or $G_{\alpha o}$ antibody did not alter the response to either $G_{\alpha i2}$ antibody or $G_{\alpha o}$ antibody alone (49 ± 2% inhibition for $G_{\alpha i2}$ plus G_β antibody and 43 ± 9% inhibition for $G_{\alpha o}$ plus G_β antibody).

G proteins and PLC-β isozyme coupled to μ and κ receptors. A limited number of experiments were done to

TABLE 3

Effect of antibodies to PLC-β isozymes (10 μg/ml) and G protein subunits (10 μg/ml) on DPDPE-stimulated PLC-β activity in plasma membranes and on Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction in permeabilized intestinal muscle cells

DPDPE-stimulated PLC-β activity was expressed as increase in inositol phosphate formation above basal levels (608 ± 75 cpm/mg-min). Ins(1,4,5)P_3 mass was expressed as increase above basal levels (3.03 ± 0.2 pmol/10^6 cells). Ca^{2+} release was expressed as percentage decrease in steady state Ca^{2+} content (2.38 ± 0.41 nmol/10^6 cells) in cells preloaded with $^{45}Ca^{2+}$. Contraction was measured by scanning micrometry and expressed as percentage decrease in control cell length (103 ± 2 μm). Values are mean ± standard error of four or five experiments.

	PLC activity	Ins(1,4,5)P_3	Ca^{2+} release	Contraction
	Δcpm/mg-min	*Δpmol/10^6 cells*	*Δ% in cell Ca^{2+}*	*Δ% in length*
DPDPE (1 μM)	2067 ± 224	5.6 ± 0.5	30.5 ± 3.0	26.8 ± 1.8
+PLC-β1 antibody	1966 ± 164	5.3 ± 0.6	31.0 ± 1.3	25.8 ± 2.0
+PLC-β2 antibody	2066 ± 144	5.6 ± 0.3	31.2 ± 2.6	27.3 ± 1.6
+PLC-β3 antibody	663 ± 103[a]	1.1 ± 0.2[a]	4.9 ± 1.6[a]	4.6 ± 0.7[a]
+PLC-β4 antibody	2043 ± 186	5.6 ± 0.8	30.4 ± 2.6	28.0 ± 0.2
+$G_{\alpha i1}$ antibody	2115 ± 198	5.3 ± 0.6	27.6 ± 2.1	24.6 ± 2.8
+$G_{\alpha i1\text{-}2}$ antibody	2044 ± 287	5.4 ± 0.4	28.7 ± 0.7	24.1 ± 0.6
+$G_{\alpha i3}$ antibody	2043 ± 242	5.1 ± 0.2	29.9 ± 2.5	23.5 ± 2.5
+$G_{\alpha o}$ antibody	2133 ± 149	NT	30.1 ± 3.7	26.9 ± 2.9
+G_β antibody	691 ± 77[a]	1.8 ± 0.4[a]	5.2 ± 1.0[a]	3.7 ± 1.2[a]
+$G_{\alpha q/11}$ antibody	1983 ± 290	5.0 ± 0.5	29.1 ± 1.6	23.5 ± 2.0

[a] $p < 0.01$.

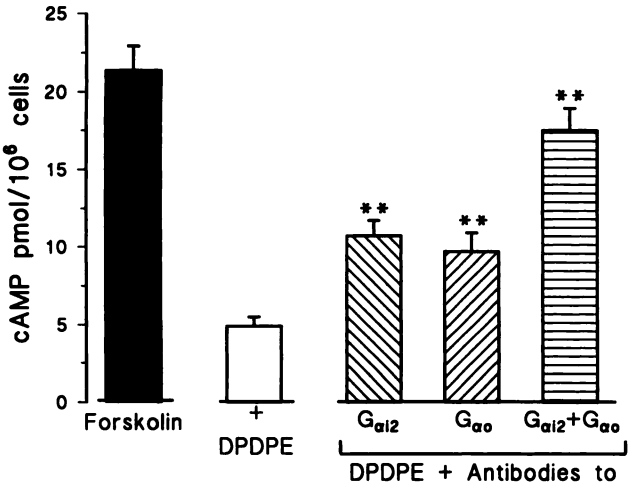

Fig. 5. Effect of G protein antibodies on DPDPE-induced inhibition of forskolin-stimulated cAMP formation in intestinal muscle cells. Permeabilized muscle cells were treated with 10 μM forskolin alone or with 1 μM DPDPE for 60 sec. Measurements were made with or without preincubation for 1 hr with G protein antibodies (10 μg/ml). Control forskolin-stimulated cAMP formation was 20.6 ± 2.0 pmol/10^6 cells above basal levels of 4.6 ± 0.3 pmol/10^6 cells. The effects of $G_{\alpha i2}$ and $G_{\alpha o}$ antibody (each 10 μg/ml) were additive, causing complete reversal of cAMP inhibition. $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha s}$, and $G_{\alpha q/11}$ antibodies had no effect (data not shown). Values are mean ± standard error of three experiments. **, Significant reversal of DPDPE-induced inhibition of cAMP formation, $p < 0.01$.

determine whether μ and κ receptors were coupled in similar fashion to δ receptors. The μ receptor agonist DAMGO (1 μM) and the κ receptor agonist U-69,593 (1 μM) caused maximal contraction of permeabilized intestinal circular muscle cells (25.0 ± 2.0% and 26.6 ± 0.8% decrease in cell length, respectively) that was not significantly different from that caused by 1 μM DPDPE (Tables 3 and 4). Pretreatment of the cells with 800 ng/ml PTX inhibited contraction induced by DAMGO and U-69,593 by 78 ± 8% and 80 ± 8%, respectively (Table 4). Pretreatment of permeabilized muscle cells with 10 μg/ml G_β antibody inhibited contraction induced by DAMGO and U-69,593 by 86 ± 3% and 83 ± 5%, respectively (Table 4); pretreatment with all other G protein antibodies had no significant effect.

Pretreatment with 10 μg/ml PLC-β3 antibody inhibited contraction induced by DAMGO and U-69,593 by 85 ± 4% and 78 ± 2%, respectively (Table 4). Pretreatment with PLC-β1, PLC-β2, and PLC-β4 antibodies had no significant effect (range, 5 ± 4% to 9 ± 4% inhibition) (Table 4). The magnitudes of inhibition of DAMGO-, U-69,593-, and DPDPE-induced contraction by PTX, G_β antibody, and PLC-β3 antibody were closely similar. The results implied that μ and κ receptors also were coupled to activation of PLC-β3 via the βγ subunits of PTX-sensitive G proteins.

As noted above, DAMGO and U-69,593, like DPDPE, stimulated GTPγS binding to the α subunits of both G_{i2} and G_o but not to the α subunits of other G proteins (Table 1). The results implied that the βγ subunits responsible for activation of PLC-β3 were derived from both G_{i2} and G_o. The involvement of both G proteins was supported by analysis of inhibition of forskolin-stimulated cAMP formation. DAMGO (1 μM) and U-69,593 (1 μM) inhibited forskolin-stimulated cAMP formation in permeabilized muscle cells by 75 ± 3% and 73 ± 6%, respectively (Fig. 6). The inhibition induced by both agonists was reversed by PTX to 16 ± 14% and 19 ± 17%. Pretreatment of the cells with 10 μg/ml $G_{\alpha i1\text{-}2}$, $G_{\alpha i2}$, and $G_{\alpha o}$ antibody reversed DAMGO-induced inhibition of forskolin-stimulated cAMP formation to 48 ± 6%, 49 ± 6%, and 56 ± 6%, respectively, and U-69,593-induced inhibition to 46 ± 8%, 54 ± 10%, and 55 ± 6%, respectively (Fig. 6). The effect of a combination of $G_{\alpha i2}$ and $G_{\alpha o}$ antibodies was additive, reversing DAMGO-induced inhibition to 19 ± 3% and U-69,593-induced inhibition to 16 ± 9% (Fig. 6). Pretreatment with 10 μg/ml $G_{\alpha i3}$, $G_{\alpha i1}$, $G_{\alpha q/11}$, or G_β had no significant effect on DAMGO- or U-69,593-induced inhibition of forskolin-stimulated cAMP formation (range, 72 ± 4% to 78 ± 2%; not significantly different from control).

TABLE 4

Effect of PTX, PLC-β antibodies, and antibody to G_β on DAMGO- and U-69,593-induced contraction in permeabilized intestinal muscle cells

Contraction was measured by scanning micrometry and expressed as percentage decrease in control cell length (103 ± 2 μm). Values are mean ± standard error of four or five experiments.

	Contraction	
	DAMGO (1 μM)	U-69,593 (1 μM)
	% decrease in cell length	
Control	25.0 ± 2.0	26.6 ± 0.8
+PTX (800 ng/ml)	5.2 ± 2.0[a]	5.4 ± 0.7[a]
+PLC-β1 antibody (10 μg/ml)	25.7 ± 2.8	28.9 ± 0.9
+PLC-β2 antibody (10 μg/ml)	24.1 ± 4.0	27.8 ± 3.5
+PLC-β3 antibody (10 μg/ml)	3.7 ± 1.0[a]	5.9 ± 0.6[a]
+PLC-β4 antibody (10 μg/ml)	27.0 ± 1.1	27.5 ± 1.1
+G_β antibody (10 μg/ml)	3.4 ± 0.7[a]	4.2 ± 1.2[a]

[a] $p < 0.01$.

Discussion

PLC-β and G protein antibodies were used in the current study to demonstrate a unique pattern of signaling by opioid μ, δ, and κ receptors in intestinal smooth muscle that differs from that in a variety of native and transformed cells (16, 17, 21, 23, 24). A similar approach using the same panels of antibodies was previously used to identify the G proteins and PLC-β isozymes activated by cholecystokinin and adenosine A_1 receptors in intestinal smooth muscle (29, 33). Smooth muscle cells in this region express a full spectrum of G proteins ($G_{q/11}$, G_s, G_{i1}, G_{i2}, G_{i3}, and G_o) and PLC-β isozymes (PLC-β1, PLC-β2, PLC-β3, and PLC-β4). The opioid receptors in intestinal circular muscle were coupled to two PTX-sensitive G proteins, G_{i2} and G_o; the α subunits of both G proteins inhibited adenylyl cyclase, whereas their βγ subunits activated PLC-β3 to cause PI hydrolysis, Ins(1,4,5)P_3-dependent Ca^{2+} release, and muscle contraction.

Evidence for the signaling cascade initiated by opioid receptors in intestinal muscle can be summarized as follows. 1) DPDPE (δ receptor agonist) stimulated Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction; inhibited forskolin-stimulated cAMP formation in dispersed muscle cells; and stimulated PI hydrolysis in smooth muscle plasma membranes. All of the effects were blocked by pretreatment of the cells with PTX. 2) DPDPE-stimulated Ins(1,4,5)P_3 formation, Ca^{2+} release, and contraction in permeabilized muscle cells and PI hydrolysis in plasma membranes were selectively blocked by G_β antibody and PLC-β3 antibody; contractions

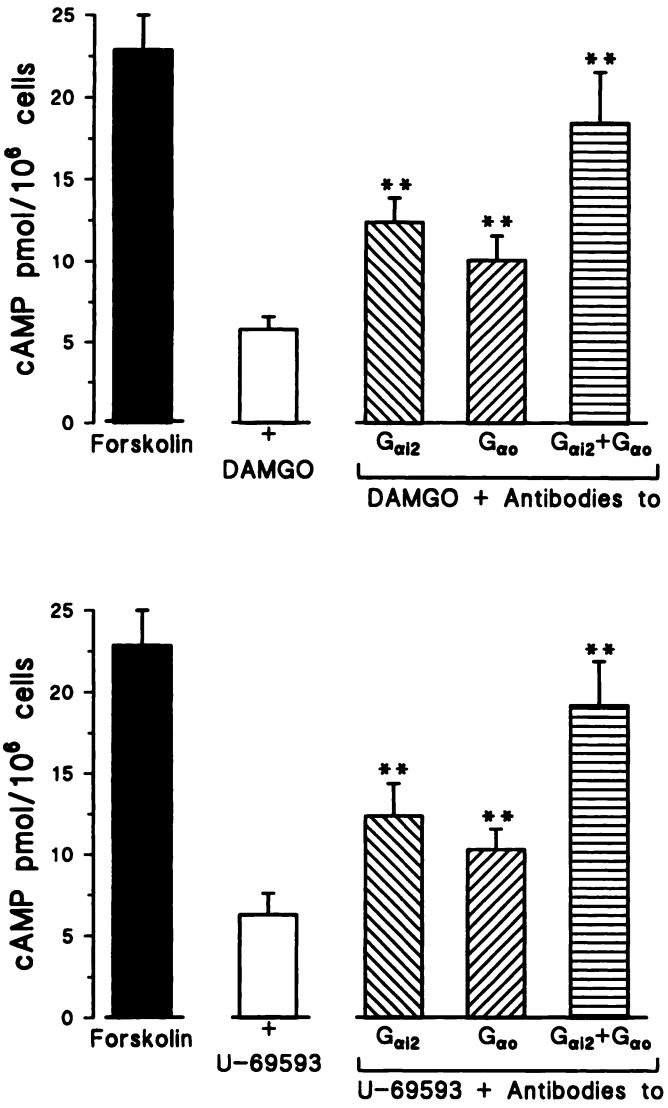

Fig. 6. Effect of G protein antibodies on DAMGO- and U-69,593-induced inhibition of forskolin-stimulated cAMP formation in intestinal muscle cells. Permeabilized muscle cells were treated with 10 μM forskolin alone and with 1 μM DAMGO or 1 μM U-69,593 for 60 sec. Measurements were made with or without preincubation for 1 hr with G protein antibodies (10 μg/ml). Control forskolin-stimulated cAMP formation was 22.9 ± 3.2 pmol/10^6 cells above basal levels of 5.1 ± 0.7 pmol/10^6 cells. The effects of $G_{\alpha i2}$ and $G_{\alpha o}$ antibody (each 10 μg/ml) were additive. $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha s}$, and $G_{\alpha q/11}$ antibodies had no significant effect (data not shown). Values are mean ± standard error of three experiments. **, Significant reversal of DAMGO- and U-69,593-induced inhibition of cAMP formation, $p < 0.01$.

stimulated by DAMGO (μ receptor agonist) and U-69,593 (κ receptor agonist) were also blocked by G_β antibody and PLC-β3 antibody. 3) Inhibition of forskolin-stimulated cAMP formation induced by δ, μ, and κ receptor agonists was additively blocked by $G_{\alpha i2}$ and $G_{\alpha o}$ antibodies. 4) The δ, μ, and κ receptor agonists stimulated the binding of [^{35}S]GTPγS to $G_{\alpha i2}$ and $G_{\alpha o}$ but not to the α subunits of other G proteins.

The pattern of inhibition of adenylyl cyclase and activation of PLC-β3 by opioid receptors in intestinal smooth muscle differed from that induced by other receptor types (e.g., adenosine A_1 and somatostatin 3 receptors) coupled to inhibitory G proteins. Adenosine A_1 receptors are coupled to activation of PLC-β3 via both the $\beta\gamma$ and α subunits of G_{i3} and to inhibition of adenylyl cyclase via the α subunit of G_{i3} (29), whereas somatostatin 3 receptors are coupled to activation of PLC-β3 via the $\beta\gamma$ subunits of G_{i1} and G_o and to inhibition of adenylyl cyclase via the α subunits of both G proteins (35). Despite the differences in G protein coupling, opioid receptors share important features with adenosine and somatostatin receptors and with other receptors (e.g., muscarinic M_2 and α_2-adrenergic receptors) coupled to inhibitory G proteins. These include activation of K^+ channels and inhibition of Ca^{2+} channels in central and peripheral neurons and results in inhibition of neuronal activity and transmitter release (1, 3, 13). The effects on K^+ and Ca^{2+} channels are mediated directly by G proteins and do not involve the participation of diffusible products of effector enzymes (3, 13).

Activation of PLC-β3 by opioid receptors in smooth muscle conforms to a pattern of preferential activation of this isozyme by the $\beta\gamma$ subunits of inhibitory G proteins (36–38). However, direct G protein-dependent activation of a PLC-β isozyme has not been demonstrated in transformed neural cell lines expressing native or cloned opioid receptors, with one notable exception (i.e., activation of an unidentified PLC-β in undifferentiated NG108-15 cells during periods of rapid growth) (23, 25). In differentiated NG108-15 cells or other neuroblastoma cell lines, however, stimulation of PI hydrolysis by opioid agonists seems to be an indirect consequence of stimulation of Ca^{2+} influx via a receptor-specific process involving the participation of one or more inhibitory G proteins, the type and abundance of which depend on the cell line (23–25). On the other hand, in transfected Chinese hamster ovary and COS cells and in some neurons, the same inhibitory G proteins mediate inhibition of PLC-β activity (27). In marked contrast, freshly dispersed intestinal muscle cells provide an unequivocal demonstration of the ability of the three main opioid receptor types, δ, μ, and κ, to stimulate PI hydrolysis and Ca^{2+} mobilization by activating a specific PLC-β isozyme, PLC-β3, via the $\beta\gamma$ subunits of two PTX-sensitive G proteins, G_{i2} and G_o. In this respect, opioid receptors conform to a pattern shared by several receptor types that involves $G_{\beta\gamma}$-dependent activation of PLC-β3 and G_α-dependent inhibition of adenylyl cyclase, the main difference being the type and number of inhibitory G proteins yielding these subunits (29, 35). The fact that G_α antibodies did not affect activation of PLC-β3 by opioid or somatostatin receptors suggests that the G_α and G_β antibodies bound to the subunits after dissociation of the trimeric G protein.

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