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Regulation of Adenylyl Cyclase Type V/VI in Smooth Muscle: Interplay of Inhibitory G Protein and Ca^{2+} Influx

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ABSTRACT

The characteristics of inhibitory regulation of adenylyl cyclase V/VI by Ca^{2+} and G proteins were examined in dispersed gastric smooth muscle cells. The mechanisms were evoked separately, sequentially, or concurrently using ligand-gated and G protein-coupled receptor agonists and receptor-independent probes (e.g, thapsigargin). During the initial phase of agonist stimulation, α,β-methylene-ATP, UTP, and ATP inhibited forskolin-stimulated cAMP formation in a concentration-dependent fashion. Inhibition by α,β-methylene-ATP, which activates ligand-gated P_{2X} receptors, was abolished by zero Ca^{2+}, whereas inhibition by UTP, which activates P_{2Y2} receptors coupled to $G_{q/11}$ and G_{i3}, was not affected by zero Ca^{2+} but was abolished by pertussis toxin (PTX). Inhibition by ATP, which activates both P_{2X} and P_{2Y2} receptors, was not affected by zero Ca^{2+} alone; but after inhibition mediated by $G_{\alpha i3}$ was blocked with PTX, inhibition by Ca^{2+} influx was unmasked and was abolished by zero Ca^{2+}. Inhibition by cholecystokinin-8 was observed only during the phase of capacitative Ca^{2+} influx and was blocked by zero Ca^{2+}. Inhibition by UTP during this phase was not affected by zero Ca^{2+} alone; but after inhibition mediated by $G\alpha_{i3}$ was blocked with PTX, inhibition by Ca^{2+} influx was unmasked and was abolished by zero Ca^{2+}. Inhibition of adenylyl cyclase V/VI activity in smooth muscle can be mediated independently by inhibitory G proteins and Ca^{2+} influx but is exclusively mediated by inhibitory G proteins when both mechanisms are triggered.

Functional regulation of the 10 cloned isoforms of adenylyl cyclase is diverse, with no two isoforms displaying identical regulation (Cooper *et al*., 1995; Sunahara *et al*., 1996). Nevertheless, three broad categories can be distinguished comprising (1) types I and VIII, predominantly expressed in neurons and stimulated by submicromolar concentrations of Ca^{2+} and calmodulin and a more widely expressed type III, stimulated by low micromolar concentrations of Ca^{2+} (Choi *et al*., 1992a, 1992b; Xia *et al*., 1992; Cali *et al*., 1994), (2) types II, IV, and IX, which are not affected by Ca^{2+} or inhibited (in the case of types II and IV) by the GTP-binding proteins G_i and G_o (Tang and Gilman, 1992; Taussig *et al*., 1993, 1994; Premont *et al*., 1996), and (3) types V and VI, which are inhibited by G_i and G_o and by submicromolar concentrations of Ca^{2+} elicited by Ca^{2+} influx but not by Ca^{2+} release from intracellular stores (Boyajian *et al*., 1991; Yoshimura and Cooper, 1992; Chiono *et al*., 1995; Taussig and Gilman, 1995).

We have shown recently that adenylyl cyclase types V and VI, but not types II, III, or IV, are expressed in gastrointestinal smooth muscle (Murthy and Makhlouf, 1997). The cyclases are inhibited, depending on the agonist, by G_{i1}, G_{i2}, G_{i3}, and G_o. Inhibition via somatostatin sst3 receptors is mediated by G_{i1} and G_o (Murthy *et al*., 1996), whereas inhibition via opioid μ, δ, or κ receptors is mediated by G_{i2} and G_o (Murthy and Makhlouf, 1996). Inhibition via adenosine A_1 receptors, muscarinic m_2 receptors, and P_{2Y2} receptors is mediated by G_{i3} (Murthy and Makhlouf, 1995a, 1997, 1998). Stimulation of adenylyl cyclase via muscarinic m_3 receptors is mediated by the $\beta\gamma$ subunit of $G_{q/11}$; the stimulation is masked by the predominant inhibition mediated via m_2 receptors by G_{i3} (Murthy and Makhlouf, 1997).

The coexistence of receptor subtypes coupled to distinct signaling pathways is likely to elicit various patterns of regulation of adenylyl cyclase V/VI in smooth muscle. For example, stimulation of adenylyl cyclase activity by adenosine A_2 receptors coupled to G_s is attenuated by A_1 receptors coupled to inhibition of adenylyl cyclase via the α subunit of G_{i3} and to activation of PLC-β3 and IP_3-dependent Ca^{2+} release via the α and $\beta\gamma$ subunits of G_{i3} (Murthy and Makhlouf, 1995a): inhibitory regulation could be mediated by Ca^{2+} influx, inhibitory G protein, or both. Whether these inhibitory mechanisms operate in concert or are mutually exclusive has not been determined.

In the current study, we examined the characteristics of inhibitory regulation of adenylyl cyclase V/VI by Ca^{2+} and G

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ABBREVIATIONS: IP_3, inositol triphosphate; PTX, pertussis toxin; CCK-8, cholecystokinin octapeptide; PLC, phospholipase C; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PDE, phosphodiesterase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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proteins in smooth muscle. Experiments were designed in which the mechanisms were evoked separately, sequentially, or concurrently using a variety of agonists and probes. G protein-independent Ca^{2+} influx was elicited by the P_{2X} receptor agonist α,β-methylene-ATP (Fredholm *et al*., 1997; Surprenant *et al*., 1995), and G protein-dependent and -independent capacitative Ca^{2+} influx was elicited by agonists (cholecystokinin-octapeptide) and thapsigargin, respectively. Both mechanisms could be elicited concurrently or sequentially with the P_{2Y2} agonist UTP, which is coupled to both $G_{q/11}$ and G_{i3} (Harden *et al*., 1995; Nicholas *et al*., 1996; Murthy and Makhlouf, 1998), or the mixed P_{2X} and P_{2Y2} agonist ATP. PTX was used to uncouple P_{2Y2} receptors from G_{i3}. The results indicate that inhibition can be independently mediated by Ca^{2+} influx and inhibitory G proteins, but when both mechanisms are triggered, inhibition is exclusively mediated by inhibitory G proteins.

Experimental Procedures

Dispersion of smooth muscle cells. Muscle cells were isolated from the circular muscle layer of the rabbit stomach by successive enzymatic digestion, filtration, and centrifugation as described previously (Murthy and Makhlouf, 1997). Briefly, slices of gastric muscle were obtained from the body of circular muscle; the initial slices at the boundaries presumed to contain the majority of interstitial cells of Cajal were discarded. The muscle slices were incubated for 30 min at 31° in 15 ml of HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The composition of the medium was 120 mM NaCl, 4 mM KCl, 2.6 mM KH_2PO_4, 0.6 mM $MgCl_2$, 25 mM HEPES, 14 mM glucose, and 2.1% Eagle's essential amino acid mixture; no Ca^{2+} was added to the medium. The partly digested tissue was washed with 100 ml of enzyme-free medium and reincubated for 30 min, during which the cells were allowed to disperse spontaneously without tissue trituration. Suspensions of single muscle cells (~20 × 10^6 cells) were harvested by filtration through 500-μm Nitex mesh. The suspensions were centrifuged twice for 10 min at 350 × *g* to eliminate cell debris and organelles, in particular, neural membranes as shown previously using [^{3}H]saxitoxin binding (Murthy and Makhlouf, 1994). Muscle cells prepared in this fashion exclude trypan blue (95–98%) and were studied no later than 1–2 hr after dispersion. Muscle cell length ranged in length from 70 to 150 μm.

Measurement of Ca^{2+} release and uptake in dispersed smooth muscle cells. Ca^{2+} release and uptake were measured in dispersed muscle cells as described previously (Poggioli and Putney, 1982; Bitar *et al*., 1986). The muscle cells (10^7 cells in 10 ml) were incubated in a medium containing $^{45}Ca^{2+}$ (10 μCi/ml) and antimycin (10 μM), and Ca^{2+} uptake into nonmitochondrial Ca^{2+} stores was measured at intervals for 60 min when a steady state was attained (steady state $^{45}Ca^{2+}$ cell content, 2.46 ± 0.12 nmol/10^6 cells). UTP (10 μM) was added, and $^{45}Ca^{2+}$ cell content was measured at intervals for 10 min and expressed in nanomoles or percent change from steady state $^{45}Ca^{2+}$ cell content. Decrease in $^{45}Ca^{2+}$ cell content during the initial 15–30 sec reflected net Ca^{2+} release.

Measurement of cAMP in dispersed smooth muscle cells by radioimmunoassay. cAMP was measured in dispersed cells by radioimmunoassay as described previously (Murthy and Makhlouf, 1996 and 1997). Aliquots (0.5 ml) containing 10^6 cells/ml were incubated with 10 μM forskolin and the test agent in the presence of 10 μM isobutyl methylxanthine, 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°. The supernatant was extracted three times with 2 ml of 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.

Experimental design. Several experimental approaches were devised to distinguish between the effects of Ca^{2+} and inhibitory G proteins. (1) In one set of experiments, forskolin (10 μM) was added to dispersed smooth muscle cells either alone or together with a Ca^{2+}-mobilizing agonist for 60 sec, and cAMP formation during this period was measured. Measurements were made in Ca^{2+}-containing and Ca^{2+}-free medium (0 Ca^{2+} plus 1 mM EGTA) and in muscle cells preincubated for 60 min with 400 ng/ml PTX. (2) In another set of experiments, the muscle cells were treated for 5 min with a maximally effective concentration of a Ca^{2+}-mobilizing agonist so as to elicit capacitative Ca^{2+} influx and then were treated with forskolin for 60 sec. Measurements were made in Ca^{2+}-containing and Ca^{2+}-free medium and in muscle cells treated for 10 min with the PLC-β inhibitor U-73122 or for 60 min with PTX. (3) In control experiments, agonist-independent capacitative Ca^{2+} influx was elicited by treating the muscle cells with thapsigargin (2 μM) for 30 min in Ca^{2+}-free medium followed by restitution of normal Ca^{2+}; alternatively, the muscle cells were treated with ionomycin (10 μM), which induces both Ca^{2+} release and Ca^{2+} influx. Measurements of forskolin-stimulated cAMP formation were made in the presence or absence of Ca^{2+}.

Data analysis. Results were expressed as mean ± standard error and were evaluated statistically using Student's *t* test for paired or unpaired values.

Materials. [^{125}I]cAMP and $^{45}Ca^{2+}$ were obtained from DuPont-New England Nuclear (Boston, MA). HEPES was from Research Organics (Cleveland, OH). Soybean trypsin inhibitor and collagenase (type II) were from Worthington Biochemicals (Freehold, NJ). α,β-Methylene-ATP and β,γ-methylene-ATP were from Research Biochemicals (Natick, MA). PTX, vinpocetine, U-73122, and thapsigargin were from Calbiochem (San Diego, CA). All other chemicals were from Sigma Chemical (St. Louis, MO).

Results

The effect of forskolin on cAMP formation and agonist-induced Ca^{2+} release in dispersed smooth muscle cells. Forskolin caused a prompt increase in cAMP formation above basal level that attained a peak within 1 min declining slowly over a period of 10 min (Fig. 1). CCK-8 had no effect on

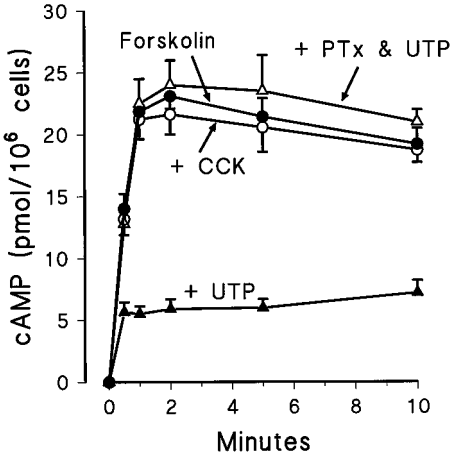

Fig. 1. Time course of forskolin-stimulated cAMP formation in dispersed smooth muscle cells. cAMP formation attained a peak 1 min after addition of forskolin (10 μM) and declined slightly over a 10-min period (●). UTP (10 μM; ▲) but not CCK-8 (1 nM; ○) inhibited cAMP formation; inhibition by UTP was reversed by preincubation of the muscle cells for 60 min with 400 ng/ml PTX (△). The results are expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.81 ± 0.42 pmol/10^6 cells). Values are mean ± standard error of three or four experiments.

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basal cAMP (basal cAMP, 4.8 ± 0.4 pmol/10^6 cells; CCK-8, 4.8 ± 0.5 pmol/10^6 cells) or on forskolin-stimulated cAMP (peak forskolin response, 21.9 ± 2.5 pmol/10^6 cells above basal level; forskolin plus CCK-8, 21.7 ± 3.8 pmol/10^6 cells). In contrast, the P_{2Y2} receptor agonist UTP inhibited forskolin-stimulated cAMP (forskolin plus UTP, 5.5 ± 0.8 pmol/10^6 cells above basal level); the inhibition by UTP was completely blocked by preincubation of the muscle cells for 60 min with 400 ng/ml PTX.

In muscle cells loaded with $^{45}Ca^{2+}$, both CCK-8 (1 nM) and UTP (10 μM) caused prompt release of Ca^{2+} from sarcoplasmic stores (34 ± 1% and 32 ± 2% decrease in steady state $^{45}Ca^{2+}$ cell content in 30 sec, respectively) followed by slower uptake into the stores over a period of 10 min (Fig. 2). Treatment of the cells with 1 μM nifedipine had no significant effect on CCK- or UTP-induced Ca^{2+} release, which occurred mainly during the first 1-min period, but it blocked Ca^{2+} reuptake, implying that capacitative Ca^{2+} influx to replenish the depleted Ca^{2+} stores is mediated by dihydropyridine-sensitive Ca^{2+} channels. The results are similar to those

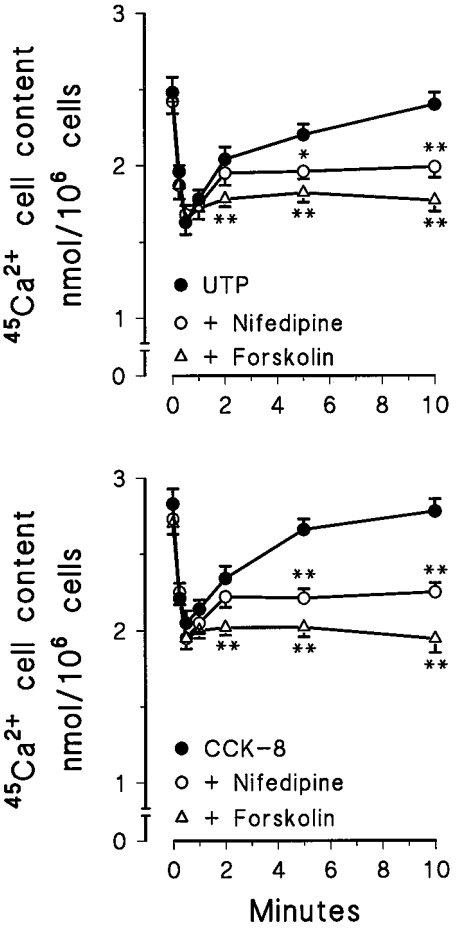

Fig. 2. Time course of Ca^{2+} release and reuptake induced by UTP and CCK-8 in smooth muscle cells. Muscle cells were incubated in a medium containing $^{45}Ca^{2+}$ (10 μCi/ml) and antimycin (10 μM), and Ca^{2+} uptake into nonmitochondrial Ca^{2+} stores was measured at intervals for 60 min when a steady state $^{45}Ca^{2+}$ cell content (2.5–2.8 nmol Ca^{2+}/10^6 cells) was attained. UTP (10 μM) or CCK-8 (1 nM) was added with or without nifedipine (1 μM) or forskolin (10 μM), and $^{45}Ca^{2+}$ cell content was measured at intervals for 10 min. Nifedipine and forskolin had no effect on Ca^{2+} release (first minute) but inhibited Ca^{2+} reuptake. Data are mean ± standard error of four experiments. *, $p < 0.05$, **, $p < 0.01$ from control levels.

obtained previously in smooth muscle cells stimulated with CCK-8 in which methoxyverapamil was shown to block Ca^{2+} reuptake into the stores (Bitar *et al.*, 1986).

The concomitant addition of forskolin had no effect on CCK- or UTP-induced Ca^{2+} release during the first minute but abolished capacitative Ca^{2+} influx into the cells. A comparison of the time course of forskolin-stimulated cAMP and that of agonist-stimulated Ca^{2+} release in the presence and absence of forskolin suggested that Ca^{2+} release did not exert an inhibitory effect on cAMP formation in smooth muscle cells expressing adenylyl cyclase V/VI. The inhibitory role of capacitative Ca^{2+} influx was examined by altering the experimental design as described subsequently.

Differential inhibition of adenylyl cyclase activity by G protein-coupled P_{2Y} receptor and ligand-gated P_{2X} receptor agonists. We have shown recently that the initial increase in $[Ca^{2+}]_i$ induced by UTP, a P_{2Y2} receptor agonist in gastric smooth muscle cells, was mediated by IP_3-dependent Ca^{2+} release, whereas the increase in $[Ca^{2+}]_i$ induced by α,β-methylene-ATP, a P_{2X1} receptor agonist in these cells, was mediated by Ca^{2+} influx via voltage-sensitive Ca^{2+} channels (Murthy and Makhlouf, 1998). The increase in $[Ca^{2+}]_i$ induced by ATP, a mixed P_{2Y2}/P_{2X1} receptor agonist in these cells, was mediated by both Ca^{2+} release and Ca^{2+} influx. The P_{2Y2} receptors were coupled to both $G_{q/11}$ and G_{i3}, and the stimulation of IP_3 formation and Ca^{2+} release resulted from concurrent activation of PLC-β1 by $G_{\alpha q/11}$ and PLC-β3 by $G_{\beta\gamma i3}$. The distinctive properties of these agonists were used to evaluate the regulation of adenylyl cyclase V/VI in dispersed gastric smooth muscle cells. Selective adenosine A_1 and A_2 receptor antagonists [1 μM DPCPX cyclopentyl-1,3-dipropylxanthine and 0.1 μM CGS-15943 (9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine] were added to the medium to prevent effects that could result from degradation of purine agonists (Murthy and Makhlouf, 1995a).

The inhibitory effects of α,β-methylene-ATP, UTP, and ATP on forskolin-stimulated cAMP formation measured during the first 60 sec of agonist stimulation were concentration dependent (Figs. 3-5). Inhibition induced by 10 μM α,β-methylene-ATP, UTP, and ATP was 39 ± 3%, 53 ± 3%, and 61 ± 5%, respectively. The percentage inhibition of cAMP by 10 μM α,β-methylene-ATP (48 ± 5%) or UTP (57 ± 6%) was not altered when measurements were done in the presence of a high concentration of IBMX (500 μM) to eliminate the possibility of degradation by Ca^{2+}-stimulated PDE1. The percentage inhibition of cAMP by α,β-methylene-ATP (47 ± 6%) or UTP (59 ± 7%) also was not altered when measurements were done in the presence of the selective PDE1 inhibitor vinpocetine (100 μM).

Inhibition of cAMP formation by α,β-methylene-ATP was completely blocked by withdrawal of Ca^{2+} from the medium (0 Ca^{2+}/1 mM EGTA) but was not affected by pretreatment of the muscle cells for 60 min with 400 ng/ml PTX (Fig. 3). Inhibition induced by combining α,β-methylene-ATP (10 μM) with CCK-8 (1 nM) (40 ± 6%) was not significantly different from that induced by α,β-methylene-ATP alone (39 ± 3%). In contrast, inhibition of cAMP formation by UTP was not affected by withdrawal of Ca^{2+} from the medium but was completely blocked by pretreatment of the muscle cells with PTX (Fig. 4). Inhibition of cAMP formation by ATP was only partly blocked by pretreatment of the muscle cells with PTX (inhibition with 10 μM ATP, 61 ± 5%; inhibition after PTX

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treatment, 38 ± 2%; $p < 0.01$ for the difference), whereas withdrawal of Ca^{2+} from the medium had no significant effect (Fig. 5). Complete blockade of inhibition, however, was achieved by pretreatment of the muscle cells with PTX and withdrawal of Ca^{2+} from the medium. The inhibition of cAMP formation by ATP after pretreatment with PTX was attributed to Ca^{2+} influx resulting from activation of P_{2X} receptors because it was completely blocked by withdrawal of Ca^{2+} from the medium.

Thus, inhibition of adenylyl cyclase activity by UTP during the first minute of agonist stimulation was exclusively mediated by a PTX-sensitive G protein, G_{i3}, whereas inhibition by α,β-methylene-ATP was exclusively mediated by Ca^{2+} influx via voltage-sensitive Ca^{2+} channels. Inhibition by ATP was mediated by both G_{i3} and Ca^{2+} influx; the effect of Ca^{2+} influx was seen only after the effect mediated by G_{i3} was blocked with PTX.

Inhibition of adenylyl cyclase activity in smooth muscle cells by agonist-dependent capacitative Ca^{2+} influx. To examine the effects of capacitative Ca^{2+} influx triggered by depletion of intracellular Ca^{2+} stores on adenylyl cyclase activity, the design of the experiments was altered as follows. The muscle cells were first treated for 5 min with the agonist so as to evoke capacitative Ca^{2+} influx, after which 10 μM forskolin was added for 1 min. The two agonists used in these experiments were CCK-8, which activates $G_{q/11}$ (Murthy and Makhlouf, 1995b), and UTP, which activates both $G_{q/11}$ and G_{i3} (Murthy and Makhlouf, 1998) in these cells.

After treatment of muscle cells for 5 min with 1 nM CCK-8 and then with 10 μM forskolin for 1 min, cAMP formation was inhibited by 32 ± 5% ($p < 0.01$, four experiments) (forskolin alone, 20.4 ± 2.4 pmol cAMP/10^6 cells; forskolin plus CCK-8,

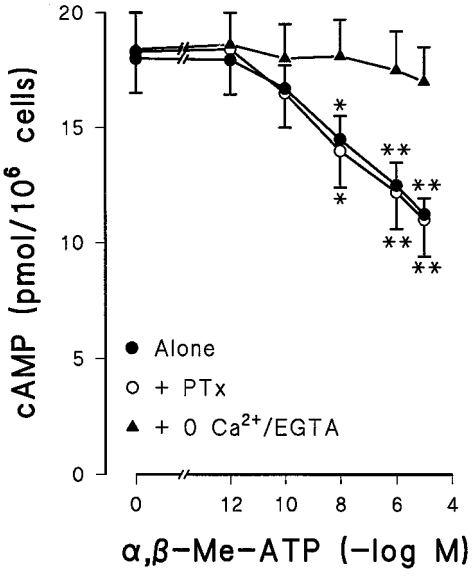

Fig. 3. Concentration-response curves for α,β-methylene-ATP-induced inhibition of forskolin-stimulated cAMP formation in smooth muscle cells. The effects of α,β-methylene-ATP on forskolin-stimulated cAMP formation in dispersed gastric smooth muscle cells were measured during the first 60 sec of agonist stimulation in the presence or absence of extracellular Ca^{2+} (0 Ca^{2+}/1 mM EGTA) and after treatment with PTX (400 ng/ml) for 1 hr. Forskolin was added either alone or together with the agonist for 1 min. The results were expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.59 ± 0.42 pmol/10^6 cells; basal level after PTX, 4.51 ± 0.34 pmol/10^6 cells; basal level in 0 Ca^{2+}, 4.57 ± 0.34 pmol/10^6 cells). Values are mean ± standard error of four experiments. *, $p < 0.02$; **, $p < 0.01$.

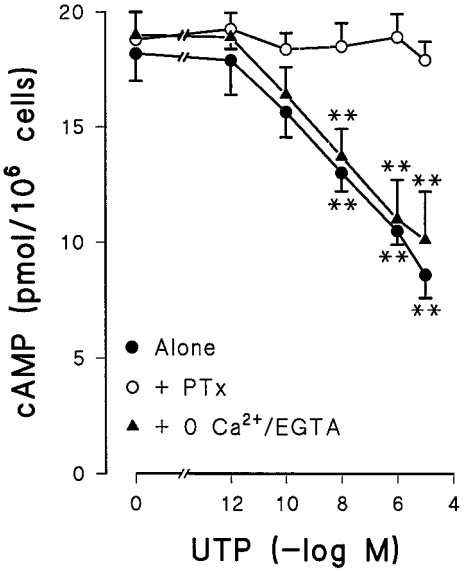

Fig. 4. Concentration-response curves for UTP-induced inhibition of forskolin-stimulated cAMP formation in smooth muscle cells. The effects of UTP on forskolin-stimulated cAMP formation in dispersed gastric smooth muscle cells were measured during the first 60 sec of agonist stimulation in the presence or absence of extracellular Ca^{2+} and after treatment with PTX for 1 hr. Forskolin (10 μM) was added either alone or together with the agonist for 1 min. The results were expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.62 ± 0.36 pmol/10^6 cells; basal level after PTX, 4.50 ± 0.31 pmol/10^6 cells; basal level in 0 Ca^{2+}, 4.70 ± 0.42 pmol/10^6 cells). Values are mean ± standard error of four experiments. **, $p < 0.01$.

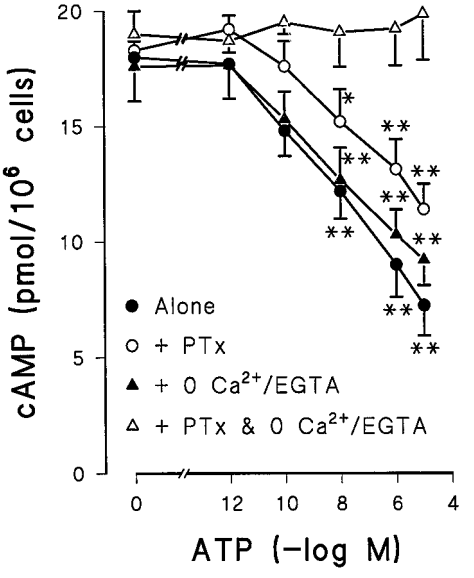

Fig. 5. Concentration-response curves for ATP-induced inhibition of forskolin-stimulated cAMP formation in smooth muscle cells. The effects of ATP on forskolin-stimulated cAMP formation in dispersed gastric smooth muscle cells were measured during the first 60 sec of agonist stimulation in the presence or absence of extracellular Ca^{2+} (0 Ca^{2+}/EGTA) and after treatment with 400 ng/ml PTX for 1 hr. Forskolin (10 μM) was added alone or together with the agonist for 1 min. The results were expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.59 ± 0.36 pmol/10^6 cells; basal level after PTX, 4.74 ± 0.34 pmol/10^6 cells; basal level in 0 Ca^{2+}, 4.57 ± 0.38 pmol/10^6 cells; basal levels in 0 Ca^{2+} after treatment with PTX, 4.80 ± 0.52 pmol/10^6 cells). Values are mean ± standard error of four experiments. *, $p < 0.05$; **, $p < 0.01$.

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13.8 ± 1.8 pmol/10^6 cells) (Fig. 6). The inhibition was abolished by withdrawal of Ca^{2+} from the medium (0 Ca^{2+}/1 mM EGTA) (forskolin plus CCK in 0 Ca^{2+}, 20.6 ± 2.3 pmol/10^6 cells). The inhibition also was abolished by 10-min treatment of the muscle cells with the PLC-β inhibitor U-73122 (20.5 ± 2.5 pmol/10^6 cells). The pattern implied that cAMP formation was inhibited by capacitative Ca^{2+} influx because the inhibition was blocked when Ca^{2+} influx was precluded by withdrawing Ca^{2+} from the extracellular medium or by preventing IP_3-dependent Ca^{2+} release and, thus, depletion of Ca^{2+} stores.

After treatment of muscle cells for 5 min with UTP (10 μM) and then with 10 μM forskolin for 1 min, cAMP formation was inhibited by 65 ± 4% ($p < 0.001$, four experiments) (forskolin alone, 20.6 ± 2.3 pmol cAMP/10^6 cells; forskolin plus UTP, 7.1 ± 0.9 pmol/10^6 cells) (Fig. 7). The inhibition of cAMP formation was not affected by withdrawal of Ca^{2+} from the medium (8.1 ± 0.8 pmol/10^6 cells; 60 ± 4% inhibition) but was partly blocked by pretreatment of the muscle cells for 60 min with PTX (14.0 ± 1.2 pmol/10^6 cells; 28.6 ± 3.5% inhibition; $p < 0.01$; four experiments). The inhibition was completely blocked by pretreatment of the muscle cells for 60 min with PTX followed by 5-min treatment with UTP in Ca^{2+}-free medium (19.9 ± 2.3 pmol/10^6 cells; 3 ± 2% inhibition; p = NS). Inhibition also was completely blocked by pretreatment of the muscle cells for 60 min with PTX followed by treatment with both U-73122 (10 μM) and UTP (20.5 ± 1.9 pmol/10^6 cells; 1 ± 5% inhibition, p = NS). In comparing the effects of UTP and CCK-8, it is worth noting that U-73122 eliminates all IP_3-dependent depletion of the Ca^{2+} stores, thereby precluding capacitative Ca^{2+} influx, whereas PTX eliminates only IP_3 formation mediated by G_{i3} but not that mediated by $G_{q/11}$, thus maintaining capacitative Ca^{2+} influx.

Inhibition of adenylyl cyclase activity in smooth muscle cells by agonist-independent capacitative Ca^{2+} influx. The ability of capacitative Ca^{2+} influx to inhibit adenylyl cyclase activity was examined further using the sarcoplasmic Ca^{2+}-ATPase inhibitor thapsigargin, which depletes intracellular Ca^{2+} stores independently of receptor activation (Thastrup, 1990). The muscle cells were incubated for 30 min with 2 μM thapsigargin in 0 Ca^{2+} plus 1 mM EGTA, followed by restitution of control Ca^{2+} levels (2 mM), and then treated with 10 μM forskolin for 1 min. In muscle cells treated with thapsigargin followed by restitution of extracellular Ca^{2+}, forskolin-stimulated cAMP formation was inhibited by 41 ± 3% (forskolin alone, 18.4 ± 2.1 pmol/10^6 cells; forskolin plus thapsigargin, 11.4 ± 1.7 pmol/10^6 cells; $p < 0.01$, $n = 4$) (Fig. 8). Inhibition was not observed when the muscle cells were maintained in Ca^{2+}-free medium after treatment with thapsigargin (18.8 ± 1.6 pmol/10^6 cells).

Similar results were obtained in experiments with ionomycin, which acts as both ionophore and stimulant of Ca^{2+} release (Murthy *et al.*, 1991). The muscle cells were treated for 1 min with 10 μM ionomycin and 10 μM forskolin in the presence or absence of Ca^{2+}. In muscle cells treated with ionomycin in the presence of 2 mM Ca^{2+}, forskolin-stimulated cAMP formation was inhibited by 40 ± 4% (forskolin alone, 18.4 ± 2.1 pmol/10^6 cells; forskolin plus ionomycin in 2 mM Ca^{2+},10.9 ± 1.7 pmol/10^6 cells; $p < 0.01$, four experiments) (Fig. 8). Inhibition was not observed in the absence of Ca^{2+} (forskolin in 0 Ca^{2+}, 18.2 ± 1.8 pmol/10^6 cells; forskolin plus ionomycin in 0 Ca^{2+}, 18.0 ± 2.0 pmol/10^6 cells). The inhibitory effect of ionomycin during this short interval seemed to reflect its ability to stimulate Ca^{2+} influx.

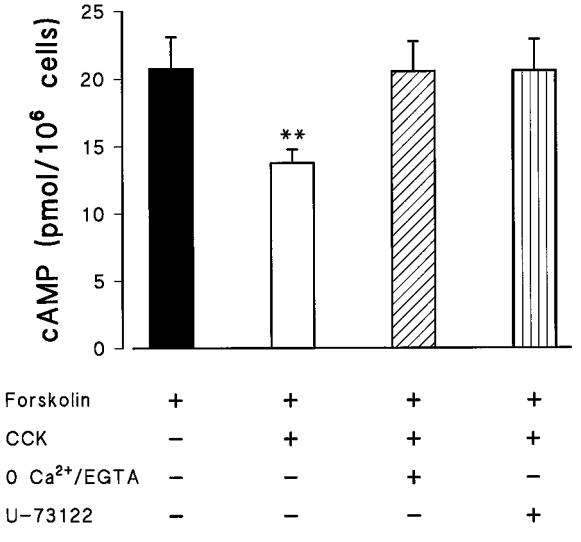

Fig. 6. Inhibition of forskolin-stimulated cAMP formation in smooth muscle cells by CCK-induced capacitative Ca^{2+} influx. The effects of capacitative Ca^{2+} influx on adenylyl cyclase activity were determined after the smooth muscle cells were treated for 5 min with CCK-8 (1 nM). The cells were treated for 1 min with 10 μM forskolin. U-73122 (10 μM) was used to inhibit PLC-β activity and IP_3-dependent Ca^{2+} release. The results were expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.51 ± 0.3 pmol/10^6 cells). Values are mean ± standard error of four experiments. **, $p < 0.01$.

Discussion

The results of the current study show the operation of two distinct mechanisms for the inhibitory regulation of adenylyl

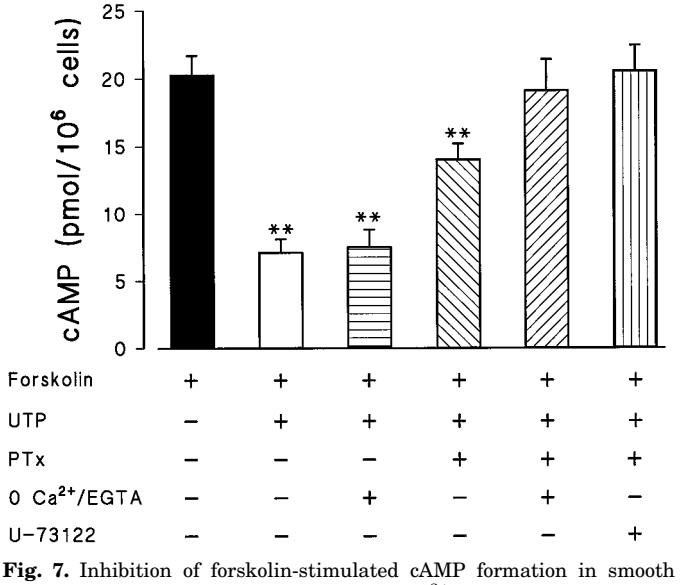

Fig. 7. Inhibition of forskolin-stimulated cAMP formation in smooth muscle cells by UTP-induced capacitative Ca^{2+} influx. To examine the effects of capacitative Ca^{2+} influx triggered by depletion of intracellular Ca^{2+} stores on adenylyl cyclase activity, the muscle cells first were treated for 5 min with the Ca^{2+} mobilizing agonist UTP (10 μM), after which 10 μM forskolin was added for 1 min. U-73122 (10 μM) was used to inhibit PLC-β activity and IP_3-dependent Ca^{2+} release. PTX was used to block the inhibition mediated by $G_{\alpha i}$. The results are expressed as pmol of cAMP/10^6 cells above basal level (basal level, 4.30 ± 0.53 pmol/10^6 cells). Values are mean ± standard error of four experiments. **, $p < 0.01$.

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cyclase types V/VI in smooth muscle cells: a G protein-dependent mechanism and a Ca^{2+}-dependent mechanism that seems to operate only in the absence of inhibitory G protein regulation. The mechanisms could be activated separately by agonists acting on G protein-coupled receptors (UTP, ATP, CCK-8) and ligand-gated receptors (α,β-methylene-ATP and ATP) and by agents that bypass receptors, such as the sarcoplasmic Ca^{2+}/ATPase inhibitor, thapsigargin, and ionomycin. The agonists acting on G protein-coupled receptors provided distinctive patterns of G protein activation that facilitated analysis of the role of each inhibitory mechanism. The Ca^{2+}-dependent mechanisms had in common the ability to induce Ca^{2+} influx via voltage-sensitive Ca^{2+} channels and did not involve activation of a Ca^{2+}-stimulated PDE1.

During the initial 1-min period of agonist stimulation that coincided with Ca^{2+} release from intracellular stores, UTP, which activates P_{2Y2} receptors coupled to $G_{q/11}$ and G_{i3} in visceral and vascular smooth muscle (Pacaud *et al.*, 1995; Murthy and Makhlouf, 1998), inhibited forskolin-stimulated cAMP formation in a concentration-dependent fashion; the inhibition was blocked by PTX but not by 0 Ca^{2+}, implying that it was mediated exclusively by $G_{\alpha i3}$. CCK-8, which activates receptors coupled to $G_{q/11}$ only, had no effect on cAMP formation. α,β-Methylene-ATP, which selectively activates ligand-gated P_{2X} receptors in smooth muscle cells, causing membrane depolarization and dihydropyridine-sensitive Ca^{2+} influx (Murthy and Makhlouf, 1998), also inhibited cAMP formation, but in contrast to UTP, the inhibition was blocked by 0 Ca^{2+} but not by PTX, implying that it was mediated exclusively by Ca^{2+} influx via voltage-sensitive Ca^{2+} channels.

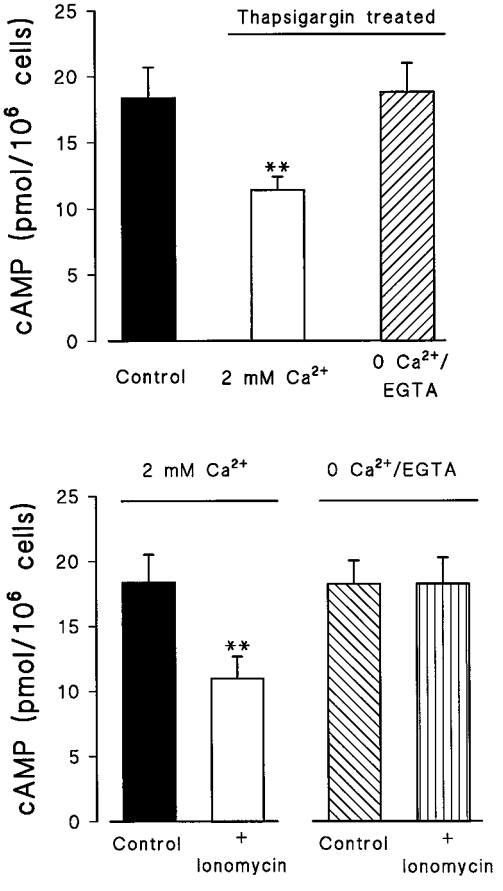

Fig. 8. Inhibition of forskolin-stimulated cAMP formation in smooth muscle cells by thapsigargin- and ionomycin-induced Ca^{2+} influx. *Top,* cAMP was measured before and after treatment with the sarcoplasmic Ca^{2+}/ATPase inhibitor thapsigargin. Muscle cells were incubated for 30 min with 2 μM thapsigargin in 0 Ca^{2+}/1 mM EGTA and then treated with 10 μM forskolin for 1 min in either the presence of 2 mM Ca^{2+} or absence of Ca^{2+}. *Bottom,* The muscle cells were treated for 1 min with 10 μM ionomycin and 10 μM forskolin in the presence or absence of Ca^{2+}. The results were expressed as pmol of cAMP/10^6 cells above basal levels (basal level, 4.62 ± 0.49 pmol/10^6 cells; basal level after thapsigargin treatment, 4.76 ± 0.31 pmol/10^6 cells; basal level in 0 Ca^{2+}/1 mM EGTA, 4.70 ± 0.35 pmol/10^6 cells). Values are mean ± standard error of four experiments. **, $p < 0.01$.

The effect of ATP, which activates both G protein-coupled P_{2Y2} and ligand-gated P_{2X} receptors, demonstrated the preferential operation of the inhibitory mechanism mediated by G_{i3}. Withdrawal of Ca^{2+} from the medium had no effect on ATP-induced inhibition of cAMP formation. However, when the inhibitory effect mediated by G_{i3} was blocked with PTX, the inhibitory effect of Ca^{2+} influx mediated by P_{2X} receptors was unmasked and could be blocked by withdrawal of Ca^{2+} from the medium. The pattern implied that when both mechanisms were elicited by different receptors, adenylyl cyclase activity was preferentially inhibited by the G protein.

Preferential inhibition by G protein also was observed with UTP during the period of capacitative Ca^{2+} influx, that is, 5 min after exposure to the agonist. Inhibition of forskolin-stimulated cAMP formation during this period was mediated by G_{i3} and could be blocked by PTX but not by withdrawal of Ca^{2+}. However, after G_{i3}-mediated inhibition was blocked with PTX, inhibition by capacitative Ca^{2+} influx was unmasked and could be blocked by withdrawal of Ca^{2+} from the medium. This pattern also implied that inhibition of adenylyl cyclase activity was preferentially mediated by the G protein that masked or suppressed the inhibitory effect of capacitative Ca^{2+} influx. In cell lines (e.g., NCB-20) in which UTP activates P_{2Y} receptors coupled to a PTX-insensitive G protein, inhibition of cAMP formation was mediated by capacitative Ca^{2+} influx (Garritsen *et al.*, 1992).

The independent inhibitory effect of capacitative Ca^{2+} influx was seen to best advantage after 5-min treatment with CCK-8 or 30-min treatment with thapsigargin to deplete the Ca^{2+} stores where inhibition of forskolin-stimulated cAMP formation was abolished by withdrawal of Ca^{2+} from the medium. Similar inhibition was obtained after 1-min treatment with ionomycin: the effect of the ionophore that induces both Ca^{2+} influx and Ca^{2+} release was mediated by Ca^{2+} influx because it was blocked on withdrawal of Ca^{2+} from the medium.

It is worth noting that inhibition of adenylyl cyclase V/VI activity by Ca^{2+} influx in smooth muscle could be elicited whether Ca^{2+} influx was triggered by (1) activation of ligand-gated P_{2X} receptors/channels (α,β-methylene-ATP and ATP), (2) capacitative Ca^{2+} influx resulting from depletion of Ca^{2+} stores by agonists (CCK, UTP) or thapsigargin, or (3) Ca^{2+} influx via ionophore (ionomycin). Earlier studies (Bitar *et al.*, 1986) have shown that repletion of Ca^{2+} stores after agonist (CCK-8) stimulation in smooth muscle cells is mediated by Ca^{2+} influx via voltage-sensitive Ca^{2+} channels; this notion was confirmed in the current study with both CCK-8 and UTP as agonists (Fig. 2). More recent studies have shown that activation of P_{2X} receptors results in membrane depolarization and Ca^{2+} influx via dihydropyridine-sensitive Ca^{2+} channels (Murthy and Makhlouf, 1998). These chan-

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nels seem to be the preferred route for inhibition of adenylyl cyclase V/VI in smooth muscle as they are in cardiac muscle, which expresses the same adenylyl cyclase isoforms (Yu *et al*., 1993; Cooper *et al*., 1995; Gao *et al*., 1997). The Ca^{2+} channels are colocalized with adenylyl cyclase in the plasma membrane of cardiac myocytes, providing a structural basis for the ability of Ca^{2+} influx to regulate adenylyl cyclase (Gao *et al*., 1997). The strict requirement for regulation by Ca^{2+} influx seems to prevail for other isoforms of adenylyl cyclase (e.g., types I, III, VIII) that are stimulated by Ca^{2+} (Fagan *et al*., 1996). Membrane colocalization and functional interplay of adenylyl cyclases and Ca^{2+} channels seem to be maintained even when the cyclases are expressed heterologously. However, neither the mechanism of inhibition of adenylyl cyclase by Ca^{2+} nor the mechanism by which concurrent inhibition by G protein precludes inhibition by Ca^{2+} influx have been defined. The absence of binding sites for Ca^{2+} or calmodulin on adenylyl cyclase seems to preclude competitive interplay between the α subunit of inhibitory G proteins and Ca^{2+}.

In summary, inhibition of adenylyl cyclase V/VI activity in smooth muscle can be mediated by inhibitory G proteins or Ca^{2+} influx independently of whether the latter is elicited by activation of ligand-gated or G protein-coupled receptors. When both mechanisms are triggered concurrently, inhibition is exclusively mediated by inhibitory G proteins.

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References

Bitar KN, Bradford PG, Putney JW Jr, and Makhlouf GM (1986) Source of activator calcium in guinea pig and human gastric muscle cells. *Am J Physiol* **250:**G280–G286.

Boyajian CL, Garritsen A, and Cooper DMF (1991) Bradykinin stimulates Ca^{2+} mobilization in NCB-20 cells leading to direct inhibition of adenylyl cyclase: a novel mechanism for inhibition of cAMP production. *J Biol Chem* **266:**4992–5003.

Cali JJ, Zwaagstra JC, Mons N, Cooper DMF, and Krupinski J (1994) Type VIII adenylyl cyclase-α Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *J Biol Chem* **269:**12190–1195.

Chiono M, Mahey R, Tate G, and Cooper DMF (1995) Capacitative Ca^{2+} entry exclusively inhibits cAMP synthesis in C6–2B glioma cells. *J Biol Chem* **270:**1149–1155.

Choi EJ, Wong ST, Hinds TR, and Storm DR (1992) Calcium and muscarinic agonist stimulation of type I-adenylyl cyclase in whole cells. *J Biol Chem* **267:**12440–12442.

Choi EJ, Xia ZG, and Storm DR (1992) Stimulation of type-III olfactory adenylyl cyclase by calcium and calmodulin. *Biochemistry* **31:**6492–6498.

Cooper DMF, Mons N, and Karpen JF (1995) Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature (Lond)* **374:**421–424.

Fagan KA, Mahey R, and Cooper DMF (1996) Functional co-localization of transfected Ca^{2+}-stimulatable adenylyl cyclases with capacitative Ca^{2+} entry sites. *J Biol Chem* **271:**12438–12444.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U, and Williams M (1997) Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* **18:**79–82.

Gao T, Puri TS, Gerhardstein BL, Chien AJ, Green RD, and Hosey MM (1997) Identification and subcellular localization of the subunits of L-type calcium channels and adenylyl cyclase in cardiac myocytes. *J Biol Chem* **272:**19401–19407.

Garritsen A, Zhang Y, and Cooper DMF (1992) Purinergic receptor regulation of signal transduction in NCB-20 cells. *Mol Pharmacol* **41:**743–749.

Harden TK, Boyer JS, and Nicholas RA (1995) P_2-purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* **35:**541–579.

Murthy KS, Coy DC, and Makhlouf GM (1996) Somatostatin receptor-mediated signaling in smooth muscle: activation of PLC-β3 by $G_{\beta\gamma}$ and inhibition of adenylate cyclase by $G_{\alpha i1}$ and $G_{\alpha o}$. *J Biol Chem* **271:**23458–23463.

Murthy KS, Grider JR, and Makhlouf GM (1991) IP_3-dependent Ca^{2+} mobilization in circular but not longitudinal muscle cells of intestine. *Am J Physiol* **261:**G937–G944.

Murthy KS and Makhlouf GM (1994) Vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide-dependent activation of membrane-bound NO synthase in smooth muscle mediated by pertussis toxin-sensitive G_{i1-2}. *J Biol Chem* **269:**15977–15980.

Murthy KS and Makhlouf GM (1995a) Adenosine A_1 receptor-mediated activation of phospholipase C-β3 in intestinal muscle: dual requirement for α and $\beta\gamma$ subunits of G_{i3}. *Mol Pharmacol* **47:**1171–1179.

Murthy KS and Makhlouf GM (1995b) Functional characterization of phosphoinositide-specific phospholipase C-β1 and -β3 in intestinal smooth muscle. *Am J Physiol* **269:**C969–C978.

Murthy KS and Makhlouf GM (1996) 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. *Mol Pharmacol* **50:**870–877.

Murthy KS and Makhlouf GM (1997) Differential coupling of muscarinic m_2 and m_3 receptors to adenylyl cyclases V/VI in smooth muscle: concurrent m_2-mediated inhibition via $G\alpha_{i3}$ and m_3-mediated stimulation via $G\beta\gamma_q$. *J Biol Chem* **272:**21317–21324.

Murthy KS and Makhlouf GM (1998) Co-expression of ligand-gated P_{2X} and G protein-coupled P_{2Y} receptors in smooth muscle: Preferential activation of P_{2Y} receptors coupled to PLC-β1 via $G\alpha_{q/11}$ and to PLC-β3 via $G\beta\gamma_{i3}$. *J Biol Chem* **273:**4695–4704.

Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden TK (1996) Uridine nucleotide selectivity of three phospholipase C-activating P_2 receptors: identification of a UDP-selective, a UTP selective and an ATP- and UTP-specific receptor. *Mol Pharmacol* **50:**224–229.

Pacaud P, Malam-Souley R, Loirand G, and Desgranges C (1995) ATP-rises $[Ca^{2+}]_i$ via different P_2-receptor subtypes in freshly isolated and cultured aortic myocytes. *Am J Physiol* **269:**H30–H36.

Poggioli J and Putney JW Jr (1982) Net calcium fluxes in rat parotid acinar cells evidence for a hormone-sensitive calcium pool in or near the plasma membrane. *Pflueg Arch Eur J Physiol* **292:**239–243.

Premont RT, Matsuoka I, Mattei MG, Pouille Y, Defer N, and Hanoune J (1996) Identification and characterization of a widely expressed form of adenylyl cyclase. *J Biol Chem* **271:**13900–13907.

Sunahara RK, Dessauer CW, and Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol* **36:**461–480.

Surprenant A, Buell G, and North RA (1995) P_{2X} receptors bring new structure to ligand-gated ion channels. *Trends Neurosci* **18:**224–229.

Tang WJ and Gilman AG (1991) Type specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science (Washington DC)* **254:**1500–1503.

Taussig R and Gilman AG (1995) Mammalian membrane-bound adenylyl cyclases. *J Biol Chem 270:*1–4.

Taussig R, Quarmby LM, and Gilman AG (1993) Regulation of purified type I and type II adenylyl cyclases by G protein $\beta\gamma$ subunits. *J Biol Chem* **268:**9–12.

Taussig R, Tang WJ, Hepler JR, and Gilman AG (1994) Distinct patterns of regulation of mammalian adenylyl cyclases. *J Biol Chem* **269:**6093–6100.

Thastrup O (1990) Role of Ca^{2+}-ATPase in regulation of cellular Ca^{2+} signalling as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. *Agents Actions* **29:**8–15.

Yoshimura M and Cooper DMF (1992) Cloning and expression of Ca^{2+}-inhibitable adenylyl cyclase from NCB-20 cell. *Proc Natl Acad Sci USA* **89:**6716–6720.

Yu HJ, Ma H, and Green RD (1993) Calcium entry via L-type calcium channels acts as a negative regulator of adenylyl cyclase activity and cyclic AMP levels in cardiac myocytes. *Mol Pharmacol* **44:**689–693.

Xia ZG, Choi EJ, Wang F, and Strom DR (1992) The type I calmodulin-sensitive adenylyl cyclase is neural specific. *J Neurochem* **60:**305–311.

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