Differential Coupling of Muscarinic m₂ and m₃ Receptors to Adenylyl Cyclases V/VI in Smooth Muscle

CONCURRENT m₂-MEDIATED INHIBITION VIA Gₐi3 AND m₃-MEDIATED STIMULATION VIA Gβγ₄

(Received for publication, February 27, 1997, and in revised form, June 18, 1997)

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Muscarinic m₂ and m₃ receptors couple preferentially to inhibition of adenyl cyclase, whereas m₁, m₅, and m₆ receptors couple preferentially to activation of phospholipase C-β and in some cells to stimulation of cAMP. Smooth muscle cells were shown to express adenyl cyclases types V and/or VI. Acetylcholine (ACh) stimulate basal cAMP (35 ± 3%) induced by ACh in dispersed muscle cells was accentuated by 4-DAMP or Gbg₄a. Binding to Gbg₄a antibody was inhibited by the m₃ receptor antagonist, 4-DAMP, and binding to Gbg₄a antibody was inhibited by the m₂ receptor antagonist, N,N’-bis[2-(methoxyphenyl)methyl]amino[hexyl]-1,8-octanediamine tetrahydrochloride (methoctramine). The decrease in cAMP caused only an increase in cAMP that was abolished by 4-DAMP or Gbg₄a antibody. In muscle cells where only m₃ receptors were preserved by selective receptor protection, ACh caused an accentuated decrease in cAMP to increase above basal level (+28 ± 5 to +32 ± 6%); the increase in cAMP was abolished by 4-DAMP or Gbg₄a antibody. In conclusion, m₂ receptors in smooth muscle cells coupled to inhibition of adenyl cyclases V/VI via Gbg₄a and m₃ receptors couple to activation of the enzymes via Gβγ₄i11.

Although functional regulation of adenyl cyclases is diverse, three broad categories can be distinguished comprising types I, III, and VIII, types II, IV, and probably VII, and types V and VI. All adenyl cyclases are activated by the diterpene, forskolin, and the α subunit of Gbg₄a. Types I and VIII, which are expressed exclusively in neurons, are stimulated by submicromolar concentrations of Ca²⁺ and calmodulin, whereas type III, which is more widely expressed, is stimulated by low micromolar concentrations of Ca²⁺ (13–16); type I is effectively inhibited by Gbgα but only moderately inhibited by Gbgα and Gbgβ (17–21). Types II and IV are not stimulated by Ca²⁺/calmodulin or inhibited by Gbg but are stimulated by Gbgγ (19, 21–24). Stimulation by Gbgγ, initially thought to be conditional on concurrent stimulation by Gbgα, is now viewed as highly synergistic, with only modest stimulation by Gbgγ alone (21). Types V and VI are inhibited by Gbg and by submicromolar concentrations of cytosolic Ca²⁺ elicited by capacitative Ca²⁺ influx but not by Ca²⁺ release from sarcoplasmic stores (24, 25–28); inhibition by Gbg or stimulation by Gbgγ remains uncertain. Both types V and VI contain consensus sequences for phosphorylation and exhibit feedback inhibition, by cAMP-dependent protein kinase (29, 30).

The expression of adenyl cyclase isoforms in smooth muscle has not been determined. The regulatory pattern suggested by our previous studies in gastrointestinal smooth muscle is consistent with the presence of types V and/or VI. Agonist-induced cAMP formation is mediated by Gbgα (31, 32), and forskolin-stimulated cAMP formation is inhibited, depending on the agonist, by Gbgα, Gbgα, and Gbgα (33–35). Thus, inhibition induced by somatostatin (acting via sstr3) is mediated by Gbgα and Gbgα (33), and inhibition induced by opioid agonists (acting via μ, δ, and κ receptors) is mediated by Gbgα and Gbgα (34); inhibition induced by adenosine (acting via A₁ receptors) is mediated by Gbgα but not by Gbgα (35). Forskolin-stimulated cAMP formation is inhibited in feedback fashion by cAMP-dependent protein kinase (36). Phorbol esters have no effect on basal or forskolin-stimulated cAMP formation (37). However, inhibition of cAMP formation induced by agonists acting via Gbgα (but not Gbgα) is partly reversed by concomitant activation of PKC; the effect reflects selective PKC-dependent phosphorylation of Gbgα and Gbgα but not Gbgα or Gbgα (37).

It is well established that the muscarinic receptors, m₂ and m₃, are preferentially coupled to inhibition of adenyl cyclase; the odd-numbered receptors, m₁, m₅, and m₆, are preferentially coupled to phosphoinositide hydrolysis but, in some cells, can

Complementary DNA clones encoding the full sequences of eight isoforms of mammalian adenylyl cyclase (types I–VIII) and the partial sequences of two additional isoforms (types IX and X) have been isolated (1–10). The amino acid sequences (range 1064–1248 residues) are arranged in two cassettes of six transmembrane-spanning domains (9–11). Overall homology is most evident between types II and IV and types V and VI. All isoforms contain consensus sequences for activation of Gbgα. Four isoforms (types II, IV, VI, and X) have been isolated (1–10). The amino acid sequences and the partial sequences of two additional isoforms (types IX and X) have been isolated (1–10). The amino acid sequences are homologous to the corresponding domains of membrane-bound homodimeric and soluble heterodimeric guanylyl cyclases (11, 12). Structural homology among the various isoforms is most evident between types II and IV and types V and VI. All the isoforms are expressed in the brain, and types V and VI are the predominant isoforms in the periphery (8–11).

* This work was supported by Grant DK-28300 from the NIDDK of the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PKC, protein kinase C; 4-DAMP, 4-diphenylacetoxyl V-methylpiperidined; ACh, acetylcholine; methoctramine, N,N’-bis[2-(methoxyphenyl)methyl]amino[hexyl]-1,8-octanediamine tetrahydrochloride; PTx, pertussis toxin; GTPγS, guanosine-5’-O-(3-thio) triphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
also increase the levels of cAMP (38–44). The mechanisms responsible for the increase in cAMP are likely to reflect the type of adenylyl cyclase expressed in various cells. In cells expressing predominantly Ca\(^{2+}\)/calmodulin-sensitive adenylyl cyclases types I, III, and VIII, activation could result from Ca\(^{2+}\) mobilization and activation of PKC (43). This, however, is unlikely in cells expressing predominantly Ca\(^{2+}\)/calmodulin-insensitive types II and IV or in cells expressing types V and VI that are inhibited by physiological levels of Ca\(^{2+}\) (10, 28). In the present study, we show that types V and/or VI, the isoforms of adenylyl cyclase expressed in smooth muscle cells, are inhibited by m\(_2\) receptors via the \(\alpha\) subunit of G\(_{q/11}\) and concurrently activated by m\(_3\) receptors via the \(\beta\gamma\) subunits of G\(_{q/11}\).

**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 (31, 33). Briefly, muscle strips were incubated for 30 min at 31 °C 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\(_2\)PO\(_4\), 25 mM HEPES, 14 mM glucose, and 2.1% Eagle’s essential amino acid mixture. After washing, the tissues were re-incubated in the same medium for 30 min. The digested tissue was washed with enzyme-free medium, and the cells were allowed to disperse spontaneously for 30 min. Suspensions of single muscle cells were harvested by filtration through 500-μm Nitex mesh. The suspensions were centrifuged twice for 10 min at 350 × g.

In experiments with G protein antibodies, the cells were permeabilized as described previously (31, 33) by 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 NaHPO\(_4\), 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.

**Radioligand Binding to Muscarinic Receptors in Dispersed Muscle Cells**—Radioligand binding to dispersed muscle cells was done as described previously (33). Muscle cells were suspended in HEPES medium containing 1% bovine serum albumin. Tricarboxylic aliquots (0.5 ml) of cell suspension (10\(^6\) cells/ml) were incubated for 15 min with 1 nM \[^3H\]scopolamine alone or in the presence of acetylcholine, methoctramine, or 4-DAMP. Bound and free radioligand were separated by rapid filtration under reduced pressure through 0.45-μm polycarbonate Nuclepore filters followed by repeated washing (4 times) with 3 ml of ice-cold HEPES medium containing 0.2% bovine serum albumin. Non-specific binding was measured as the amount of radioactivity associated with the muscle cells in the presence of 10 μM unlabeled ligand. Specific binding was calculated as the difference between total and non-specific binding (mean ± S.E. 33 ± 6%). IC\(_{50}\) values were calculated from competition curves using the P. fit program (Biosoft; Elsevier Publishing, Cambridge, UK).

**Measurement of cAMP in Dispersed Muscle Cells by Radioimmunoassay**—cAMP was measured in dispersed cells by radioimmunoassay as described previously (33, 34). Aliquots (0.5 ml) containing 10\(^5\) cells/ml were incubated with 0.1 μM acetylcholine, and the reaction was terminated after 60 with 6% cold trichloroacetic acid (v/v). The mixture was centrifuged at 2,000 × g for 15 min at 4 °C. The supernatant was extracted three times with 2 ml of diethyl ether and lyophilized. The samples were reconstituted for radioimmunoassay in 500 ml 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.

**Selective Protection of Muscarinic Receptors**—A technique of selective receptor protection was used to determine the presence and function of m\(_2\) and m\(_3\) receptors. The technique was previously used to determine the co-existence and function of opioid \(\mu\), \(\delta\), and \(\kappa\) receptors (45), 5-hydroxytryptamine 5-HT\(_1\) and 5-HT\(_3\) receptors (32), histamine H\(_3\) and H\(_4\) receptors (46), and tachykinin NK\(_1\), NK\(_2\), and NK\(_3\) receptors (47). The technique involves protection of one receptor type with a selective antagonist followed by inactivation of all unprotected receptors by brief treatment with a low concentration of N-ethylmaleimide. In the present study, the selective m\(_2\) receptor antagonist, methoctramine (10 nM), and m\(_3\) receptor antagonist, 4-DAMP (10 nM), were used in separate experiments to protect m\(_2\) and m\(_3\) receptors, respectively. Freshly dispersed muscle cells were incubated with one antagonist at 31 °C for 2 min followed by addition of 5 μM N-ethylmal-
Muscle cells in which only \( m_2 \) receptors were preserved lost the ability to contract (EC\(_{50} \) 0.4 nM in muscle cells expressing one receptor type). The concentration of acetylcholine that elicited maximal contraction (0.1 \( \mu \)M) abolished \([3H]\)scopolamine binding. The radioligand binding and pharmacological studies in dispersed gastric smooth muscle cells confirmed the selectivity of the antagonists and the validity of the receptor protection technique.

**Expression of Adenylyl Cyclase Isoforms in Gastric Smooth Muscle**—Western blot analysis of homogenates derived from dispersed gastric smooth muscle cells using antibodies to adenylyl cyclase type II, III, IV, and a common antibody to types V and VI only (Fig. 2). In contrast, homogenates from rat brain disclosed the presence of type V and type VI only (Fig. 2). The selective expression of types V and/or type VI in smooth muscle conforms to the predominant expression of these two types in peripheral tissues (8–11). The properties of adenylyl cyclase in gastric and intestinal smooth muscle are consistent with the properties of types V and VI; both isozymes are inhibited by Go and Ga\(_{\alpha}\) (unlike types II and IV) (33, 34) and are not stimulated by Ca\(^{2+}\) or calmodulin (unlike types I, III, and VIII) (10, 28).

**Identification of \( G \) Proteins Coupled to Muscarinic \( m_2 \) and \( m_3 \) Receptors**—Incubation of solubilized muscle cell membranes with acetylcholine (0.1 \( \mu \)M) and \( [35S] \)GTP\(_S\) (60 nM) for 20 min caused a significant, time-dependent increase in the binding of \( [35S] \)GTP\(_S\)–Ga complexes to wells pre-coated with specific antibody to Go\(_{\alpha_{11}}\) and Go\(_{\alpha_3}\) but not to wells pre-coated with antibodies to Go\(_{\alpha_6}\), Go\(_{\alpha_{1-2}}\), or Go\(_{\alpha_5}\) (Fig. 3 and Table I). The increase in bound radioactivity reflected acetylcholine-dependent activation of the dissociated \( \alpha \) subunits of Go\(_{\alpha_{11}}\) and Go\(_{\alpha_3}\) by \( [35S] \)GTP\(_S\)-G\(_{\alpha}\) complexes to wells pre-coated with specific antibody to Go\(_{\alpha_6}\) or Go\(_{\alpha_{1-2}}\). The increase in bound radioactivity corresponded to type V/VI only was detected in smooth muscle.
which G to wells pre-coated with G were raised, were used to block the binding of GTP binding of GTP antagonist (see Table I). Values are means ± S.E. of four experiments.

Peptides I and II, comprising the G protein sequences against which the Gaq and Gα11 antibodies, respectively, were raised, were used to block the binding of GTPγS-Gα complexes to the corresponding antibody. Peptide I inhibited the binding of GTPγS-Gα complexes to Gα11 antibody in a concentration-dependent fashion, whereas peptide II had no effect (Fig. 3 and Table I). It is noteworthy that the peptides inhibited the control binding of GTPγS-Gα complexes to the corresponding antibody as well as the increase in binding induced by acetylcholine.

**Dual cAMP Response of Dispersed Smooth Muscle Cells to Acetylcholine**—Acetylcholine (0.1 μM) caused a significant 35 ± 5% decrease in basal cAMP levels of dispersed gastric muscle cells (basal level, 3.9 ± 0.6 pmol/10^6 cells) (Fig. 4). Neither 4-DAMP nor methoctramine alone had any effect on basal cAMP (4.5 ± 0.4 pmol/10^6 cells). Pretreatment of the cells with 0.1 μM methoctramine converted the decrease induced by acetylcholine to a significant 32 ± 6% increase above basal level (Fig. 4); the increase in the presence of methoctramine was inhibited in a concentration-dependent fashion by 4-DAMP (EC_{50} 0.5 nM) and abolished by 0.1 μM 4-DAMP (Figs. 4 and 5). Pretreatment of the cells for 1 h with 200 ng/ml PTx so as to uncouple m2 receptors from Gα3 also converted the acetylcholine-induced decrease in cAMP to a significant 36 ± 5% increase above basal level; the increase in cAMP was abolished by 0.1 μM 4-DAMP (Fig. 4).

Conversely, pretreatment of the cells with 0.1 μM 4-DAMP accentuated the decrease in cAMP induced by acetylcholine (−63 ± 6% versus control response −35 ± 5% with acetylcholine alone; p < 0.01); the decrease was reversed in a concentration-dependent fashion by methoctramine (EC_{50} 1 nM) and abolished by 0.1 μM methoctramine (Figs. 5 and 6). The accentuated decrease in cAMP in the presence of 0.1 μM 4-DAMP was abolished by pretreatment of the cells with PTx (Fig. 6). The pattern of response to acetylcholine reflected concurrent inhibition of cAMP mediated by m2 receptors and stimulation mediated by m3 receptors.

The protein kinase C inhibitor, calphostin C (1 μM), had no effect on the increase in cAMP induced by acetylcholine in the presence of methoctramine or PTx (+37 ± 4 and +39 ± 5%, respectively, versus +35 ± 5%) or the accentuated decrease in cAMP induced by acetylcholine in the presence of 4-DAMP (−59 ± 5% versus −63 ± 6%).

**Identification of G Proteins Coupled to m2 and m3 Receptors by Functional Blockade with Antibodies**—Permeabilized muscle cells were used to identify the G proteins coupled to m2 and m3 receptors by functional blockade with G protein antibodies. Basal cAMP and the decrease in cAMP induced by acetylcholine were not affected by permeabilization (basal level, 4.2 ± 0.5 pmol/10^6 cells; acetylcholine-induced decrease of cAMP, −33 ± 3%). Pretreatment of permeabilized muscle cells with methoctramine, PTx, or Gα3 antibody (10 μg/ml) converted the decrease in cAMP to an increase above basal level (+27 ± 5, +35 ± 5, and +28 ± 5%, respectively) (Fig. 7). Preincubation of the cells for 1 h with Gα3 antibody (10 μg/ml) abolished the increase in cAMP induced by all three agents (Fig. 7), and preincubation with antibodies to Gαq/11 or Gα11–2 (each 10 μg/ml) had no effect (range of response +27 ± 7 to +30 ± 6%). The increase in cAMP induced by Gα13 antibody was also abolished by 4-DAMP (2 ± 5%).

Preincubation of permeabilized muscle cells for 1 h with Gα3 antibody (10 μg/ml) accentuated the decrease in cAMP induced by acetylcholine (−51 ± 6% versus control response −35 ± 5%; p < 0.05) (Fig. 8); preincubation with antibodies to Gαq/11 or Gα11–2 had no effect (−33 ± 6% and −29 ± 3%). The accentuated decrease in cAMP induced by Gα3 antibody was abolished by methoctramine, PTx, or Gα3 antibody (Fig. 8). 4-DAMP also accentuated the decrease in cAMP induced by acetylcholine (−56 ± 4% versus control response −33 ± 5%; p < 0.01) (Fig. 8). The accentuated decrease induced by 4-DAMP was abolished by preincubation of the cells with Gα13 antibody (10 μg/ml) (Fig. 8) but not with antibodies to Gαq/11 or Gα11–2 (−53 ± 6% and −57 ± 3%).
The binding of acetylcholine-stimulated GTP-γ-S-Gαi3 complexes in smooth muscle membranes to specific G protein antibodies (Ab)

| Gαq/11 Ab | Gαq/2 Ab | Gαi3-2 Ab | Gαi3 Ab | Gαi6 Ab |
|-----------|----------|-----------|---------|---------|
| GTP-γ-S alone | 3414 ± 377 | 3287 ± 419 | 3525 ± 604 | 2477 ± 273 | 1067 ± 214 |
| Acetylcholine (0.1 μM) | 7594 ± 895 | 7948 ± 801 | 3757 ± 405 | 2512 ± 277 | 1100 ± 278 |
| Methoctramine (0.1 μM) | 7268 ± 768 | 3375 ± 345 | NT | NT | NT |
| + 4-DAMP (0.1 μM) | 3715 ± 498 | 7562 ± 580 | NT | NT | NT |
| + p-F-HHISiD (0.1 μM) | 3428 ± 342 | 7618 ± 624 | NT | NT | NT |
| + Peptide I (1 mM) | NT | 3607 ± 482 | NT | NT | NT |
| + Peptide I (1 μM) | 7433 ± 624 | 156 ± 85 | NT | NT | NT |
| + Peptide II (1 mM) | 3628 ± 568 | NT | NT | NT | NT |
| + Peptide II (1 μM) | 232 ± 178 | 7638 ± 745 | NT | NT | NT |

* Significant inhibition, p < 0.01 to 0.001.

** NT, not tested.

Table I: Binding of [35S]GTP-γ-S-Gαi3 complex to antibody-coated wells

**Fig. 4.** Effect of the selective m2 receptor antagonist, methoctramine, and pertussis toxin on the cAMP response to acetylcholine in dispersed smooth muscle cells. cAMP levels were expressed as percent change from basal levels (3.9 ± 0.6 pmol/10^6 cells). The response to ACh (0.1 μM) was measured before and after a 10-min treatment with methoctramine (0.1 μM) or a 60-min treatment with PTx (200 ng/ml). Methoctramine or PTx converted the decrease in cAMP to increase above basal level; the increase was abolished by 4-DAMP (0.1 μM). Values are means ± S.E. of four experiments.

**DISCUSSION**

The present study confirmed the co-existence of muscarinic m2 and m3 receptors on gastric smooth muscle cells and demonstrated the differential coupling of the two receptor types to adenylyl cyclase. Muscarinic m2 receptors, the predominant receptor type expressed in gastrointestinal smooth muscle (40, 53), are known to be coupled to inhibition of adenylyl cyclase via a PTx-sensitive G protein (40, 54–56). The present study identified this G protein as Gαq. In addition, the present study demonstrated a direct coupling of m2 receptors to activation of adenylyl cyclase via the βγ subunits of Gαq11. Although coupling of m3 receptors to activation of adenylyl cyclase was known to occur in some cell types (38, 41), the mechanism(s) underlying this effect had not been determined.

The evidence for the differential coupling of m2 and m3 receptors was based on a combination of experimental strategies. (a) GTP-γ-S-Gα complexes activated by m3 receptors bound selectively to Gαq11 antibodies, whereas GTP-γ-S-Gα complexes activated by m2 receptors bound selectively to Gαq antibodies. No acetylcholine-induced increase in binding to Gαq11 or Gαq antibodies could be detected. The binding of GTP-γ-S-Gα complexes to Gαq or Gαq11 antibodies was selectively blocked by peptide fragments against which these antibodies were raised. (b) Blockade of m2 receptors or their uncoupling from G proteins by PTx converted the decrease in cAMP induced by acetylcholine to increase above basal level; the increase was blocked by a selective m3 receptor antagonist and by a common antibody to Gβγ, implying that activation of adenylyl cyclase was mediated by Gβγ derived from m3-dependent activation of Gαq11. (c) Concurrent activation of adenylyl cyclase mediated by m3 receptors attenuated the predominant inhibition mediated by m2 receptors; blockade of the stimulatory effect with an m3 receptor antagonist or Gαi3 antibody accentuated the decrease in cAMP; the accentuated decrease was abolished by methoctramine, PTx, and Gαi3 antibody. (d) The results obtained in naive muscle cells expressing both receptor types were corroborated in cells where only one receptor type was preserved. In cells where only m2 receptors were preserved, acetylcholine caused an increase in cAMP (25 ± 3%), similar to that elicited in naive cells when m2 receptors were blocked with methoctramine or uncoupled with PTx, or when Gαq was blocked with Gαq11 antibody (Fig. 9). In cells where only m3 receptors were preserved, acetylcholine elicited a decrease in cAMP and PTx reversed the decrease (Fig. 9). In cells where both m2 and m3 receptors were preserved, acetylcholine elicited a decrease in cAMP and PTx reversed the decrease (Fig. 9).

The selectivity of the antagonists used for receptor protection was demonstrated by radioligand binding, and the measured IC50 values closely matched those derived from measurements.
in cells expressing cloned m2 or m3 receptors (39, 49–52). Pharmacological analysis confirmed the validity of the receptor protection technique that had previously been used to characterize a variety of receptors co-expressed on smooth muscle cells and coupled to the same or distinct signaling pathways (e.g., histamine H1 and H2 receptors (46), 5-HT2 and 5-HT4 receptors (32), adenosine A2b and A1 receptors (35), tachykinin NK1, NK2, and NK3 receptors (47), and opioid µ, δ, and κ receptors (34)). Only receptors were inactivated while post-receptor mechanisms were spared; in particular, neither basal nor forskolin-stimulated cAMP formation was affected (32, 35).

Increasing awareness of the diverse regulation of various isoforms of adenylyl cyclases requires that the proposed mechanisms for activation or inhibition of the enzymes be consistent with the properties of the adenylyl cyclase(s) expressed in a given cell type (8–11). In the present study, adenylyl cyclase types II, III, and IV could not be detected in dispersed gastric muscle cells. Type V and/or type VI was detected by Western blot analysis since the common antibody could not distinguish between the two types. The tissue expression and regulatory features of adenylyl cyclase in smooth muscle are consistent with the absence of types I and VIII which are confined to neurons (13–16), and with the absence of types II, IV, and possibly VII which are not susceptible to inhibition by Gq (11, 21–23).

The types of adenylyl cyclase (V and/or VI) expressed in smooth muscle are regulated in similar fashion and known to be inhibited by various isoforms of Gq and by feedback phosphorylation by cAMP-dependent protein kinase (25, 26, 29, 30). The response to Ach (0.1 µM) was measured before and after treatment with 0.1 µM 4-DAMP. Ach decreased basal cAMP (p < 0.01); 4-DAMP accentuated the decrease in cAMP induced by Ach (**, p < 0.01 from decrease with Ach alone); the decrease was blocked by a 10-min treatment with methoctramine (0.1 µM, Methoc) or a 60-min treatment with PTx (200 ng/ml). Values are means ± S.E. of four experiments.

### Figures

**FIG. 5.** Concentration-dependent effects of 4-DAMP and methoctramine on the cAMP response to Ach. A, muscle cells were pretreated with 0.1 µM methoctramine to elicit Ach-induced increase in cAMP, and the ability of 4-DAMP to block the increase was then determined. B, muscle cells were pretreated with 4-DAMP to accentuate the decrease of cAMP induced by Ach, and the ability of methoctramine to block the decrease was then determined. Results are expressed as percent change from basal cAMP levels. Values are means ± S.E. of three experiments.

**FIG. 6.** Effect of the selective m3 receptor antagonist, 4-DAMP, on the cAMP response to Ach in dispersed smooth muscle cells. cAMP levels were expressed as percent change from basal levels (3.9 ± 0.6 pmol/10⁶ cells). The response to Ach (0.1 µM) was measured before and after treatment with 0.1 µM 4-DAMP. Ach decreased basal cAMP (p < 0.01); 4-DAMP accentuated the decrease in cAMP induced by Ach (**, p < 0.01 from decrease with Ach alone); the decrease was blocked by a 10-min treatment with methoctramine (0.1 µM, Methoc) or a 60-min treatment with PTx (200 ng/ml). Values are means ± S.E. of four experiments.

**FIG. 7.** Effect of G protein antibodies on the cAMP response to Ach in permeabilized smooth muscle cells. cAMP was measured in permeabilized smooth muscle cells and expressed as percent change from basal levels. Permeabilization had no effect on basal levels (4.2 ± 0.5 pmol/10⁶ cells). Cells were treated for 10 min with methoctramine (0.1 µM), 60 min with PTx (200 ng/ml), or 60 min with Gαq3 antibody (10 µg/ml). Treatment with methoctramine, PTx, or Gαq3 antibody converted the decrease in cAMP induced by Ach (0.1 µM) to increase above basal level; the increase was abolished by preincubation of the cells for 60 min with Gβγ antibody (10 µg/ml). Values are means ± S.E. of four experiments. ***, p < 0.01.
coupled to \( G_{i3} \) (35, 37).

Inhibition by submicromolar concentrations of Ca\(^{2+}\) resulting from capacitative entry of Ca\(^{2+}\) is a distinctive regulatory feature of adenylyl cyclases types V and VI (10, 21); this feature, however, could not be demonstrated under our experimental conditions. The measurement of cAMP in the present study was made during the first 60 s when the concomitant rise in cytosolic Ca\(^{2+}\) induced by acetylcholine is determined by inositol 1,4,5-trisphosphate-dependent release of Ca\(^{2+}\) from sarcoplasmic stores and thus precedes store depletion and capacitative Ca\(^{2+}\) entry (57, 58). Furthermore, suppression of phosphoinositide hydrolysis and inositol 1,4,5-trisphosphate-dependent Ca\(^{2+}\) release by Goq1 antibody had no effect on the increase in cAMP mediated by m3 receptors. The concurrent activation of PKC also had no effect on adenylyl cyclase activity in gastrointestinal smooth muscle indirectly by selective phosphorylation of Goq11 and Goq2; concurrent activation of PKC attenuated the inhibition of forskolin-stimulated cAMP mediated by m3 receptors but not by adenosine A1 or muscarinic m2 receptors coupled to Goq3 (33–35).

A novel aspect of this study was the ability of Gbg\( \gamma \) derived from the dissociation of Gbg31 to activate smooth muscle adenylyl cyclase types V and/or VI. Activation was not conditional on concurrent activation of Goq3, as is the case for activation of types II and IV (11, 21). The low abundance of Gbg\( \gamma \) in peripheral tissues, particularly when derived from the dissociation of Gbg31, raises the possibility that, when expressed in smooth muscle, types V and VI may be unusually sensitive to activation by Gbg\( \gamma \) or by specific combinations of \( \beta \gamma \) subunits.

In summary, muscarinic m2 receptors are coupled to inhibition of adenylyl cyclase V and/or VI in smooth muscle via the \( \alpha \) subunit of Goq3, whereas m3 receptors are coupled to activation of the enzymes via the \( \beta \gamma \) subunits of Goq31. The cAMP response to muscarinic agonists reflects the predominant inhibitory influence of m2 receptors.

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