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Adenosine A_1 Receptor-Mediated Activation of Phospholipase C-β_3 in Intestinal Muscle: Dual Requirement for α and $\beta\gamma$ Subunits of G_{i3}

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SUMMARY

Four native and cloned adenosine receptors (ARs), designated A_1AR, A_{2a}AR, A_{2b}AR, and A_3AR, have been characterized functionally and by radioligand binding. In the present study, we have used selective antibodies to identify the G protein subunits and phospholipase C (PLC)-β isoform coupled to A_1ARs in smooth muscle membranes and permeabilized muscle cells from rabbit intestine. Immunoblot analysis disclosed the presence of a full complement of G proteins. Adenosine caused contraction of dispersed muscle cells and increases in D-*myo*-inositol-1,4,5-trisphosphate, intracellular calcium, and cAMP levels. Contraction and the increases in D-*myo*-inositol-1,4,5-trisphosphate and intracellular calcium levels were abolished by the A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine and augmented by the A_2 antagonist CGS-15943; the reverse occurred with cAMP. A selective A_1AR agonist, cyclopentyladenosine, inhibited forskolin-stimulated cAMP accumulation; the inhibition was reversed by treatment of the cells with pertussis toxin or a $G_{\alpha i3}$-specific antibody. The pattern of inhibition implied coexistence of A_1ARs and A_2ARs coupled to interactive signaling pathways, with A_2ARs mediating activation of adenylyl cyclase and A_1ARs mediating activation of PLC and inhibition of adenylyl cyclase. Adenosine-stimulated PLC activity in muscle membranes was selectively blocked by $G_{\alpha i3}$- and G_β-specific antibodies, as well as by a PLC-β_3-specific antibody, but not by antibodies to other PLC-β isoforms or G proteins. A combination of maximally effective concentrations of $G_{\alpha i3}$- and G_β-specific antibodies did not elicit greater inhibition than did either alone. In contrast, cholecystokinin-stimulated PLC activity was selectively blocked by PLC-β_1- and $G_{\alpha q/11}$-specific antibodies. Adenosine-stimulated contraction and $^{45}Ca^{2+}$ efflux in permeabilized muscle cells were also selectively blocked by $G_{\alpha i3}$-, G_β-, and PLC-β_3-specific antibodies, whereas cholecystokinin-stimulated contraction was selectively blocked by PLC-β_1- and $G_{\alpha q/11}$-specific antibodies. The results indicate that A_1ARs are coupled to PLC-β_3 via both α and $\beta\gamma$ subunits of G_{i3}.

Four ARs, designated A_1AR, A_{2a}AR, A_{2b}AR, and A_3AR, have now been cloned from various species and characterized functionally and by radioligand binding (1–5). The receptors belong to a family of G protein-coupled receptors and exhibit characteristic structural features, with membrane-spanning domains and consensus sequences for G protein binding and regulatory phosphorylation. The A_{2a}AR and A_{2b}AR appear to be coupled exclusively to activation of adenylyl cyclase, whereas the A_1AR is coupled to various signaling pathways, including activation of PLC, inhibition of phospholipase A_2 and adenylyl cyclase, and activation of K^+ and inhibition of Ca^{2+} channels (3, 6–10). The more recently cloned A_3AR appears to be coupled to activation of PLC and inhibition of adenylyl cyclase (11). The G proteins mediating the effects of A_1AR and A_3AR are PTx sensitive, but their precise identity and the role of specific subunits in mediating activation are not known (3, 7). Although $G_{\alpha i3}$ couples preferentially to A_1ARs, other PTx-sensitive G proteins (G_{i1}, G_{i2}, and G_o) also exhibit high affinity (3, 12).

The coupling of the A_1AR to PI-specific PLC is evident in DDT_1MF_2 hamster vas deferens smooth muscle cells, where adenosine and adenosine analogues stimulate PI hydrolysis and Ca^{2+} mobilization, but is less evident in transfected cells and other cell lines, where enhancement of PLC activity induced by other agonists, rather than direct activation of PLC, was observed (5, 7–10). The difference may reflect the species of transfected receptor and/or the absence of a full complement of regulatory G proteins in some cell lines.

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ABBREVIATIONS: AR, adenosine receptor; PLC, phospholipase C; CCK-8, cholecystokinin octapeptide; IP_3, D-*myo*-inositol-1,4,5-trisphosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CPA, cyclopentyladenosine; *Rp*-cAMPS, *Rp*-cyclic adenosine-3′,5′-monophosphothioate; PTx, pertussis toxin; CCK, cholecystokinin; PI, phosphoinositide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; AM, acetoxymethyl ester; PVDF, polyvinylidene difluoride.

In the present study, we have exploited the properties of dispersed smooth muscle cells isolated from the circular muscle layer of the rabbit small intestine to characterize the signaling pathways coupled to A_1ARs. In guinea pig, rabbit, and human smooth muscle cells from this layer, agonists such as CCK-8 and acetylcholine stimulate PI hydrolysis and induce IP_3-dependent Ca^{2+} release and contraction in a PTx-insensitive fashion (13–15). Our recent studies showed that CCK-stimulated PI hydrolysis in smooth muscle membranes and contraction in permeabilized circular muscle cells from guinea pig stomach and intestine are selectively blocked, in a concentration-dependent fashion, by antibodies to $G_{\alpha q/11}$ and PLC-β_1 (16, 17). We have also shown that stimulation of adenylyl cyclase and nitric oxide synthase by vasoactive intestinal peptide in gastric muscle membranes is selectively blocked by antibodies to $G_{\alpha s}$ and $G_{\alpha i1\text{-}2}$, respectively, (17), whereas inhibition of adenylyl cyclase by acetylcholine is selectively blocked by antibodies to $G_{\alpha i3}$ (18). In the present study, we have used a similar approach to identify the G protein and PLC isoform coupled to A_1ARs. Measurements of Ca^{2+} release and contraction in permeabilized circular muscle cells of rabbit intestine and PI hydrolysis in plasma membranes derived from these cells indicate that A_1ARs are coupled to PLC-β_3 via both the α and $\beta\gamma$ subunits of the PTx-sensitive G protein G_{i3}.

Materials and Methods

Preparation of dispersed muscle cells. Muscle cells were isolated as described previously (14, 15), by incubation of muscle strips from the circular muscle layer of rabbit intestine for 45 min at 31° in a HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. 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 and centrifuged twice at 350 × *g* for 10 min.

In experiments with G protein- and PLC-specific antibodies, the cells were permeabilized as described previously (13–15), 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 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 an ATP-regenerating system (5 mM creatine phosphate and 10 units/ml creatine phosphokinase).

Measurement of muscle cell contraction by scanning micrometry. Contraction was measured in intact and permeabilized muscle cells by scanning micrometry, as described previously (14, 15). A 0.25-ml aliquot of cell suspension containing 10^4 muscle cells/ml was added to 0.1 ml of medium containing 1 mM adenosine, and the reaction was terminated after 30 sec with 1% acrolein. The effect of PLC-β- and G protein-specific antibodies was determined in permeabilized muscle cells after preincubation for 1 hr with 10 μg/ml levels of each antibody. The lengths of muscle cells treated with adenosine were measured and compared with the lengths of untreated cells. Contraction was expressed as percentage decrease in mean cell length, compared with control. Control studies showed that the greatest response to adenosine was obtained at a concentration of 1 mM.

Measurement of $[Ca^{2+}]_i$ in dispersed muscle cells. $[Ca^{2+}]_i$ was measured in suspensions of intact circular muscle cells by fura-2 fluorescence, as described previously (14, 15). The cells were incubated with 2 μM fura-2/AM for 20 min, centrifuged, and resuspended in fura-2/AM-free medium for immediate measurement of $[Ca^{2+}]_i$. Two milliliters of cell suspension (10^6 cells/ml) were used for measurement of fluorescence, which was monitored at 510 nm using a Deltascan-1 fluorometer (Photon Technologies, Brunswick, NJ), with excitation wavelengths alternating between 340 and 380 nm. Autofluorescence of unloaded cells was determined in each suspension and subtracted from fluorescence of fura-2-loaded cells. $[Ca^{2+}]_i$ values were calculated from the ratios of observed, minimal, and maximal fluorescence values, under basal conditions and upon addition of test agents.

Assay for cAMP in dispersed muscle cells. cAMP levels were measured in intact and permeabilized circular muscle cells by radioimmunoassay, as described previously (19). Aliquots (0.5 ml) containing 10^6 cells/ml were incubated with 1 mM adenosine, and the reaction was terminated after 60 sec with 6% (v/v) cold trichloroacetic acid. 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 was expressed as picomoles/10^6 cells.

Radioreceptor assay for IP_3 in dispersed muscle cells. IP_3 was measured in intact circular muscle cells using the Amersham assay system, which utilizes ^{3}H-labeled IP_3 and bovine brain microsomes, as described previously (20). One milliliter of muscle cell suspension (10^6 cells/ml) was incubated with 10 mM Li^+ at 31° for 10 min, after which adenosine (1 mM) was added for 30 sec; the reaction was terminated with an equal volume of ice-cold 10% perchloric acid. After centrifugation for 10 min at 750 × *g*, the supernatant was extracted and the IP_3 content in the aqueous phase was measured. Results were computed from a standard curve and expressed as picomoles/10^6 cells.

Measurement of $^{45}Ca^{2+}$ efflux in permeabilized muscle cells. Ca^{2+} efflux was measured in permeabilized muscle cells by an adaptation of the method of Poggioli and Putney (21), as described previously (13, 14). The medium contained $^{45}Ca^{2+}$ (10 μCi/ml), antimycin (10 μM), and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (10 units/ml). ATP (1.5 mM) was added to initiate Ca^{2+} uptake, which was measured at intervals for 90 min (at which time a steady state was attained). Adenosine was then added and $^{45}Ca^{2+}$ content was measured after 30 sec. Ca^{2+} efflux was expressed as percentage decrease in steady state $^{45}Ca^{2+}$ cell content. Antibodies were added 30 min after ATP, and incubation was maintained for another 60 min. Addition of antibodies had no effect on steady state Ca^{2+} uptake (2.50 ± 0.20 versus 2.5 ± 0.3 nmol/10^6 cells).

Assay for PLC activity in muscle cell membranes. PLC activity was determined in plasma membranes that had been prelabeled with *myo*-[^{3}H]inositol, by a modification of the method of Uhing *et al.* (22). Ten milliliters of circular muscle cell suspension (2 × 10^6 cells/ml) were incubated with *myo*-[^{3}H]inositol (20 μCi/ml) for 3 hr at 31°. Cells were then centrifuged at 350 × *g* for 10 min, washed with phosphate-buffered saline, and resuspended in ice-cold Tris·HCl buffer (50 mM, pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 3 μM leupeptin, 1 μM aprotinin, and 1 μM pepstatin A. The cells were homogenized on ice and the homogenate was centrifuged at 900 × *g* for 10 min at 4°. The pellet was resuspended in the same medium and the centrifugation step was repeated. The supernatants from both centrifugations were pooled, layered on a sucrose step gradient, and centrifuged at 200,000 × *g* for 1 hr. The plasma membrane fraction was recovered from the interface between the sucrose layers and pelleted by centrifugation at 200,000 × *g* for 2 hr. The pellet was resuspended in 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 100 μg/ml leupeptin, 100 μg/ml antipain, yielding a final concentration of 2 mg of protein/ml (10,000–20,000 cpm/mg of protein), and was used immediately for assay of enzyme activity.

The PLC assay was initiated by addition of 0.4 mg of membrane

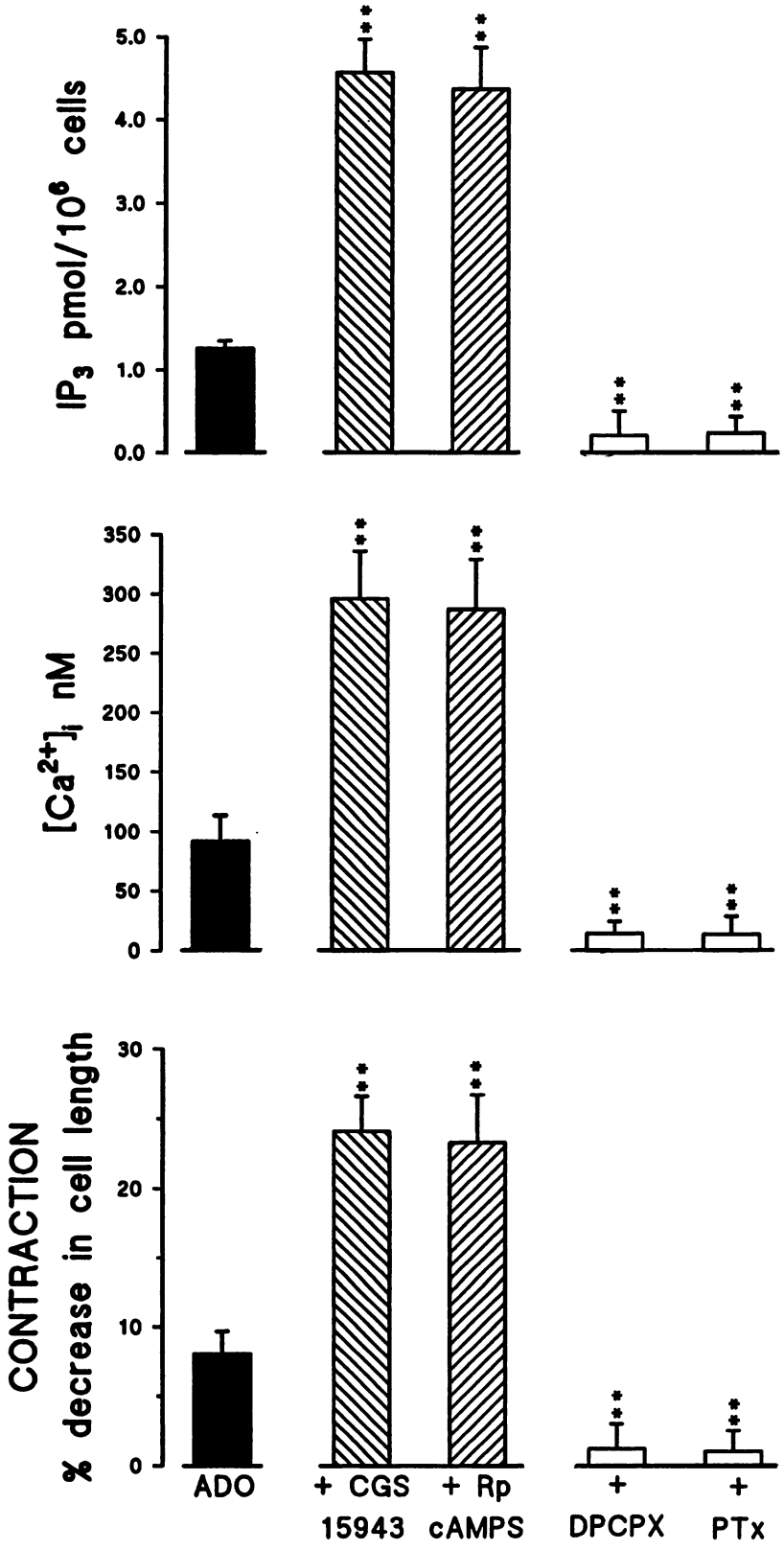

Fig. 1. Effects of CGS-15943, DPCPX, *Rp*-cAMPS, and PTx on adenosine (*ADO*)-stimulated IP_3 levels, $[Ca^{2+}]_i$, and contraction in dispersed intestinal circular smooth muscle cells. IP_3 levels were measured by radioreceptor assay and expressed as increases above the basal level (3.2 ± 0.6 pmol/10^6 cells). $[Ca^{2+}]_i$ was measured by fura-2 fluorescence and expressed as increases above the resting $[Ca^{2+}]_i$ (62 ± 6 nM). Contraction was measured by scanning micrometry and expressed as percentage decreases in cell length, compared with control (115 ± 9 μm). The cells were preincubated for 10 min with 10 nM CGS-15943, 50 nM DPCPX, or 1 μM *Rp*-cAMPS and for 60 min with PTx (200 ng/ml). Values are means ± standard errors of four experiments. **, Significant ($p < 0.01$) augmentation or inhibition of the response to adenosine alone.

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, 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% (w/v) trichloroacetic acid. 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 time 0 (80–100 cpm) was subtracted from all values. PLC activity was expressed as cpm/milligram of protein/minute.

Immunoblot analysis of G proteins in intestinal muscle membranes. Membranes were prepared from dispersed intestinal circular muscle cells as described above and were solubilized on ice

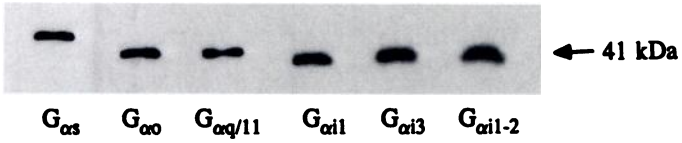

Fig. 2. Immunoblot analysis of G proteins in intestinal circular muscle. Plasma membranes isolated from dispersed intestinal circular muscle cells were solubilized with sodium cholate in Tris buffer, as described in Materials and Methods. Membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred to PVDF membranes, and then probed with G protein-specific antibodies and anti-rabbit IgG conjugated to horseradish peroxidase. The G protein bands were identified by enhanced chemiluminescence reagents.

for 1 hr in 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.5% sodium cholate. The suspension was centrifuged at 13,000 × *g* for 5 min. Solubilized membrane proteins (60–70 μg) were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membranes. The membranes were blocked with 10% dried milk in Tris-buffered saline, pH 7.6, containing 0.2% Tween-20, for 1 hr at room temperature, followed by incubation for 1 hr with different G protein-specific antibodies. After three or four washes, the PVDF membranes were incubated for 1 hr with anti-rabbit IgG conjugated to horseradish peroxidase, and the G protein bands were identified with enhanced chemiluminescence reagents.

Data analysis. Results were expressed as means ± standard errors of separate experiments and evaluated statistically using Student's *t* test for paired values.

Materials. Antibodies to $G_{\alpha 1}$, $G_{\alpha i1-2}$, $G_{\alpha i3}$, $G_{\alpha s}$, and G_β (catalogue numbers 371720, 371723, 371729, 371732, and 371738, respectively) were obtained from Calbiochem (San Diego, CA), antibody to $G_{\alpha q/11}$ (NEI-809) from NEN-DuPont, and antibodies to $G_{\alpha o}$, $G_{\alpha q/11}$, PLC-β_2, PLC-β_3, and PLC-β_4 from Santa Cruz Biotechnology (Santa Cruz, CA); the PLC-β-specific antibodies were raised against distinct sequences and did not cross-react in Western blot or immunohistochemical analyses (23). Monoclonal antibody to PLC-β_1 was obtained from Upstate Biotechnology (Lake Placid, NY). The ability of some of these antibodies (those to $G_{\alpha i1-2}$, $G_{\alpha s}$, and $G_{\alpha q/11}$) to block activation or inhibition of specific effector enzymes (PLC-β_1, nitric oxide synthase, and adenylyl cyclase) has been demonstrated in recent studies (16–18).

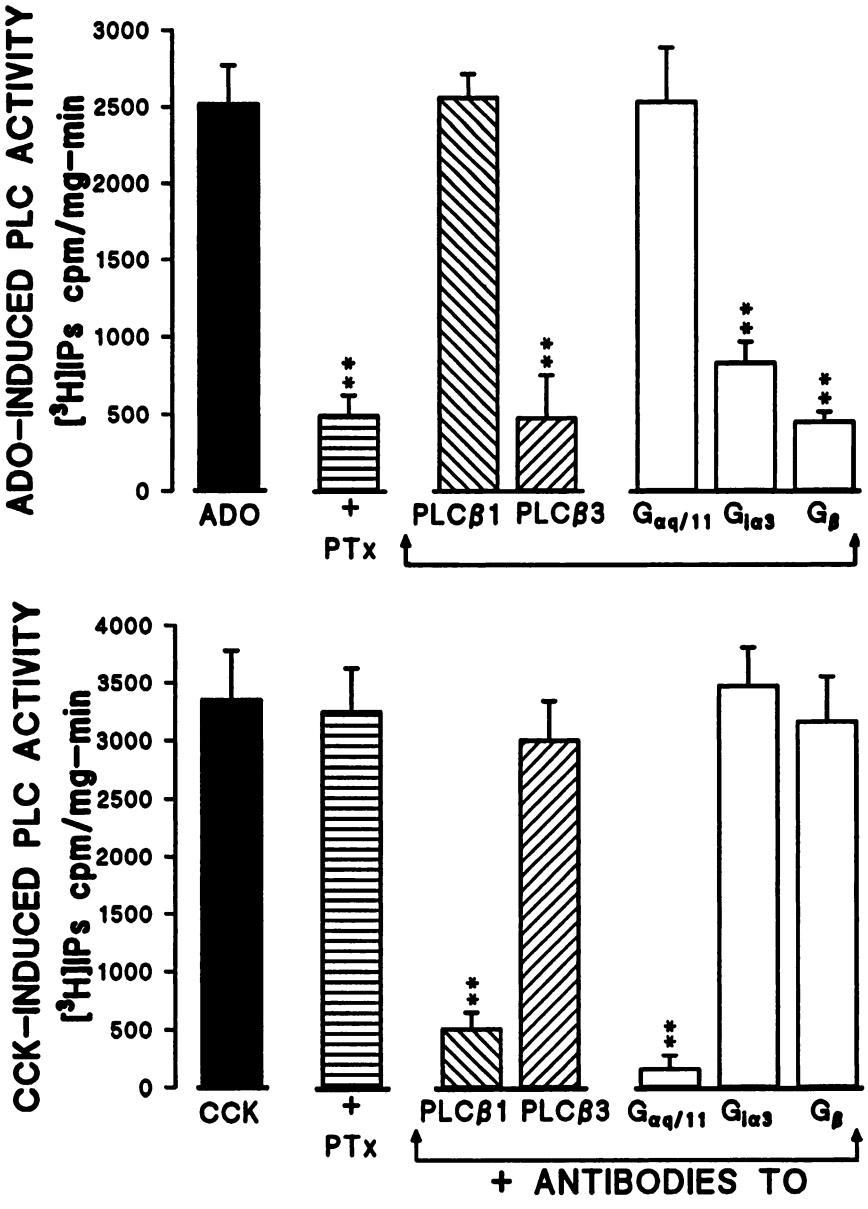

Fig. 3. Inhibition of adenosine (*ADO*)- and CCK-stimulated PLC activity in intestinal muscle membranes by antibodies to PLC-β isoforms and G protein subunits. PLC activity induced by 1 mM adenosine (*upper*) or 1 nM CCK-8 (*lower*) was measured in intestinal circular muscle plasma membranes in the presence of GTP (10 μM) and a regenerating system. The measurements were repeated in membranes that had been treated for 1 hr with 200 ng/ml PTx or 10 μg/ml levels of each antibody separately. Antibodies to PLC-β_2, PLC-β_4, $G_{\alpha o}$, $G_{\alpha s}$, and $G_{\alpha i1-2}$ had no effect on CCK- or adenosine-stimulated PLC activity and are not depicted. Results are expressed as levels of inositol phosphates ([^{3}H]IPs) above basal levels (352 ± 60 cpm/mg/min). Values are means ± standard errors of four to six experiments. **, Significant ($p < 0.001$) inhibition.

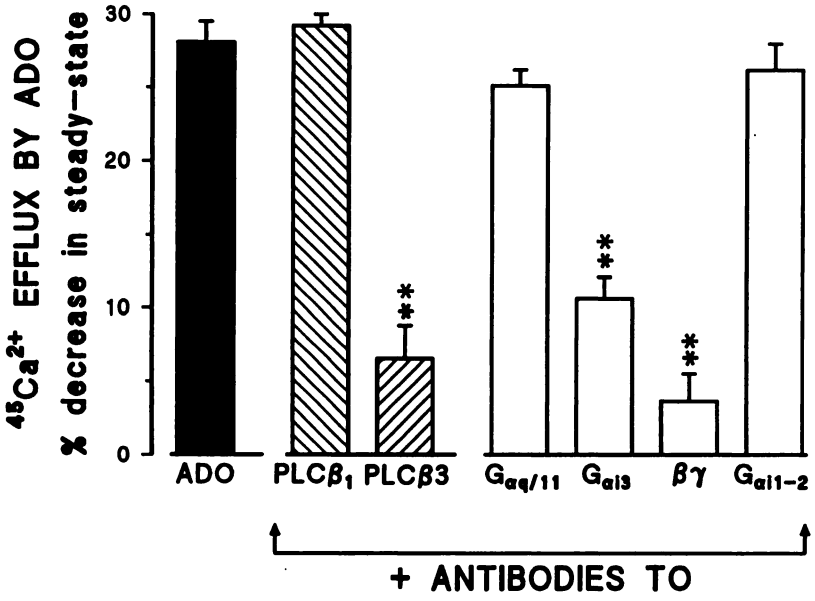

Fig. 4. Inhibition of adenosine (*ADO*)-induced $^{45}Ca^{2+}$ flux from permeabilized intestinal circular muscle cells by antibodies to PLC-β isoforms and G protein subunits. Steady state $^{45}Ca^{2+}$ uptake (2.5 ± 0.2 nmol/10^6 cells) was attained 90 min after addition of 1.5 mM ATP; adenosine (1 mM) was then added for 30 sec and $^{45}Ca^{2+}$ efflux was measured as percentage decreases in $^{45}Ca^{2+}$ cell content. Antibodies (10 μg/ml) were added 30 min after addition of ATP. CGS-15943 (10 nM) was added with adenosine to block the effect of A_2ARs. Antibodies to PLC-β_2, PLC-β_4, $G_{\alpha s}$, and $G_{\alpha o}$ had no effect and are not depicted. Values are means ± standard errors of four to six experiments. **, Significant ($p < 0.01$) inhibition.

DPCPX and CPA were obtained from Research Biochemicals (Natick, MA); CGS-15943 from Ciba-Geigy (Summit, NJ); CCK-8 from Bachem (Torrance, CA); ^{125}I-cAMP, *myo*-[^{3}H]inositol, and $^{45}CaCl_2$ from NEN-DuPont (Cambridge, MA); *Rp*-cAMPS from Biolog Life Science Institute (Bremen, Germany); anti-rabbit IgG from Bio-Rad (Melville, NY); adenosine and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO); fura-2/AM from Molecular Probes (Eugene, OR); HEPES from Research Organics (Cleveland, OH); soybean trypsin inhibitor and collagenase (type II) from Worthington Biochemicals (Freehold, NJ); IP_3 assay system from Amersham (Arlington Heights, IL); and PTx from Calbiochem.

Results

Response of intact intestinal circular muscle cells to adenosine. Adenosine (1 mM) caused contraction of dispersed intestinal circular muscle cells (8 ± 1% decrease in cell length) and increased IP_3 levels (1.3 ± 0.3 pmol/10^6 cells) and $[Ca^{2+}]_i$ (92 ± 21 nM) above resting levels (Fig. 1). Contraction, IP_3 levels, and $[Ca^{2+}]_i$ were significantly augmented after treatment of the cells for 10 min with the preferential A_2AR antagonist CGS-15943 (10 nM) or the cAMP-dependent protein kinase inhibitor *Rp*-cAMPS (1 μM), to maximal levels similar to those elicited by a maximally effective concentration of CCK-8 (14) (Fig. 1). Adenosine also increased cAMP (9.4 ± 2.6 pmol/10^6 cells above resting levels), which was abolished by CGS-15943 (0.7 ± 0.7 pmol/10^6 cells, not significant). The selective A_1AR agonist CPA inhibited forskolin-stimulated cAMP accumulation by 83 ± 4% ($p < 0.001$) (forskolin, 18.5 ± 1.5 pmol/10^6 cells; forskolin plus CPA, 3.2 ± 0.6 pmol/10^6 cells).

Treatment of the cells for 10 min with the selective A_1AR antagonist DPCPX (50 nM) or for 60 min with PTx (200 ng/ml) abolished contraction and the increases in IP_3 and $[Ca^{2+}]_i$ (Fig. 1) and significantly augmented the increase in cAMP (12.8 ± 2.4 pmol/10^6 cells and 11.0 ± 1.0 pmol/10^6 cells with DPCPX and PTx, respectively). The inhibition of forskolin-stimulated cAMP accumulation induced by the selective A_1AR agonist CPA was completely reversed by pretreatment with PTx (forskolin, 18.1 ± 1.6 pmol/10^6 cells; forskolin plus CPA, 3.2 ± 0.6 pmol/10^6 cells; forskolin plus PTx, 17.5 ± 1.9 pmol/10^6 cells).

The pattern of responses implied that adenosine activated both A_1ARs and A_2ARs. Contraction and IP_3-dependent Ca^{2+} release were mediated by A_1ARs and attenuated by concurrent activation of A_2ARs and the resultant stimulation of cAMP accumulation and cAMP-dependent protein kinase; the latter has been shown to inhibit IP_3 formation and IP_3-dependent Ca^{2+} release and contraction in this cell type (19). Conversely, the increase in cAMP levels was mediated by A_2ARs and attenuated by concurrent activation of A_1ARs coupled to inhibition of adenylyl cyclase.

Identification of the PLC-β isoform and G protein coupled to the A_1AR. A panel of G protein- and PLC-specific antibodies were used to identify the PLC isoform and G protein subunits coupled to the A_1AR. PI hydrolysis was measured in plasma membranes derived from dispersed circular muscle cells, and contraction and $^{45}Ca^{2+}$ efflux were measured in permeabilized muscle cells. A comparison was made between the responses to adenosine and to CCK-8. Immunoblot analysis of solubilized membrane fractions with the same antibodies as used for blockade of function demonstrated the presence of a full complement of G proteins; distinct bands were evident with antibodies to $G_{\alpha s}$, $G_{\alpha o}$, $G_{\alpha q}$, $G_{\alpha i1}$, and $G_{\alpha i3}$; a band elicited with the $G_{\alpha i1-2}$-specific antibody could reflect the presence of $G_{\alpha i2}$ (Fig. 2).

PLC activity (inositol phosphate formation) in membranes was increased 3-fold above basal levels upon addition of 10 μM GTP and 7-fold upon addition of 1 mM adenosine plus GTP. DPCPX inhibited adenosine-stimulated PLC activity by 83 ± 6%, and pretreatment for 1 hr with 200 ng/ml PTx inhibited activity by 81 ± 5%. Pretreatment for 1 hr with 10 μg/ml or 25 μg/ml PLC-β_3-specific antibody inhibited PLC activity by 83 ± 2% and 80 ± 5%, respectively (Fig. 3), whereas pretreatment with 10 μg/ml or 25 μg/ml levels of antibodies to PLC-β_1, PLC-β_2, and PLC-β_4 had no significant effect (range of inhibition, 3 ± 4% to 7 ± 10%; not significant).

Pretreatment for 1 hr with 10 or 25 μg/ml $G_{\alpha i3}$-specific

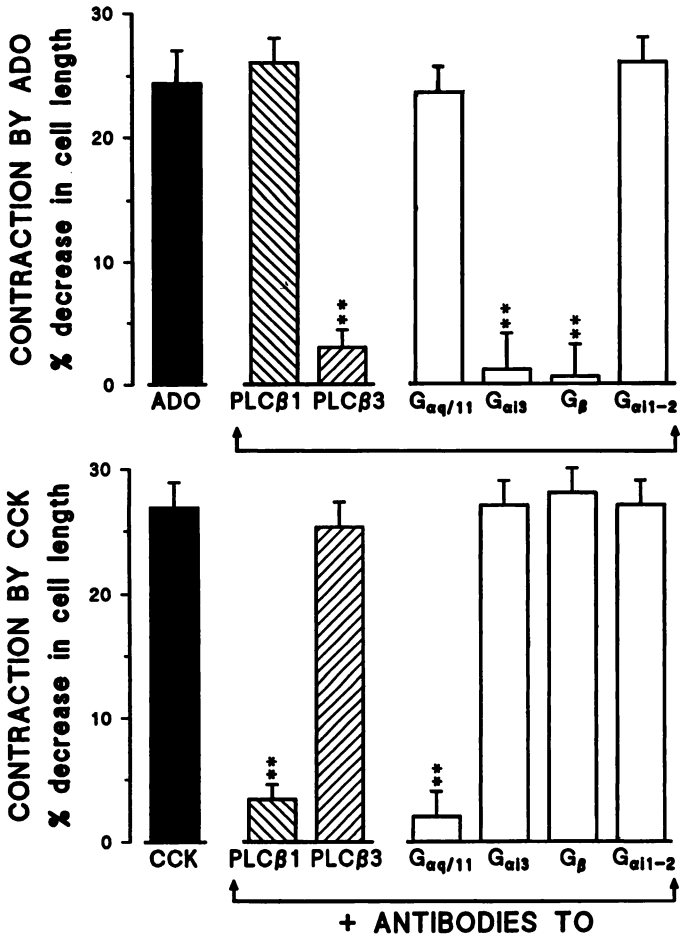

Fig. 5. Inhibition of maximal adenosine (*ADO*)- and CCK-induced contraction in permeabilized intestinal circular muscle cells by antibodies to PLC-β isoforms and G protein subunits. Permeabilized muscle cells were incubated with antibodies (10 μg/ml) for 1 hr and maximal contraction in response to 1 mM adenosine (*upper*) or 1 nM CCK-8 (*lower*) was measured by scanning micrometry, as described in Materials and Methods. Maximal contraction in response to adenosine was induced after blockade of A_2ARs with 10 nM CGS-15943. Results are expressed as percentage decreases in cell length, compared with control (102 ± 4 μm). Treatments with antibodies to PLC-β_2, PLC-β_4, and $G_{\alpha s}$ had no effect on the responses to adenosine or CCK-8 and are not depicted. Antibody to $G_{\alpha o}$ also had no effect on the response to adenosine. Values are means ± standard errors of four experiments. **, Significant ($p < 0.001$) inhibition.

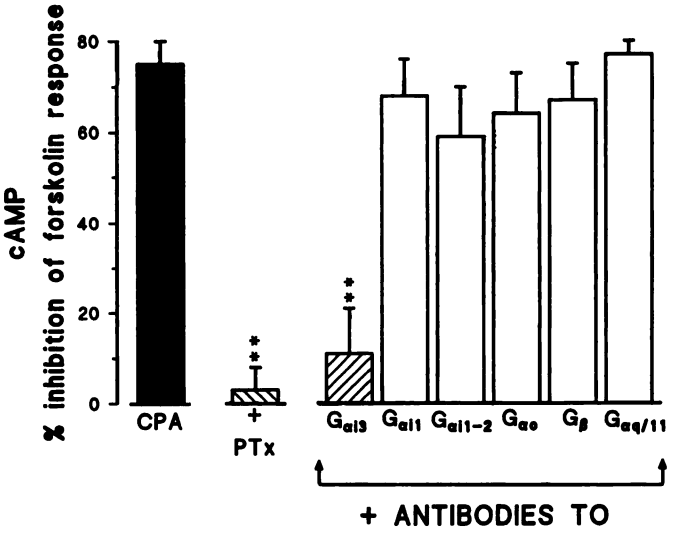

Fig. 6. Effect of G protein-specific antibodies on inhibition of forskolin-stimulated cAMP accumulation by the selective A_1AR agonist CPA. Permeabilized muscle cells were treated with 10 μM forskolin alone or in the presence of 0.1 μM CPA. Measurements were made with and without preincubation of the cells for 60 min with 200 ng/ml PTx or 10 μg/ml levels of various G protein-specific antibodies. Results are expressed as percentage inhibition of forskolin-stimulated cAMP accumulation (16.8 ± 2.1 pmol/10^6 cells above basal levels). Results are means ± standard errors of four experiments. **, Significant ($p < 0.001$) reversal of CPA-induced inhibition of cAMP accumulation.

antibody inhibited adenosine-stimulated PLC activity by 67 ± 5% and 68 ± 8%, respectively, whereas pretreatment with 10 or 25 μg/ml G_β-specific antibody inhibited PLC activity by 82 ± 3% and 78 ± 5%, respectively (Fig. 3). Pretreatment with a combination of 10 μg/ml $G_{\alpha i3}$-specific and G_β-specific antibodies did not elicit greater inhibition than that elicited by G_β-specific antibody alone (76 ± 2% for the combination versus 82 ± 3% for G_β-specific antibody alone). Pretreatment with 10 or 25 μg/ml levels of antibodies to $G_{\alpha i1\text{-}2}$, $G_{\alpha o}$, $G_{\alpha s}$, or $G_{\alpha q/11}$ had no significant effect (range of inhibition, 1 ± 4% to 10 ± 12%; not significant) (Fig. 3).

Conversely, pretreatment with PLC-β_1-specific antibody inhibited CCK-stimulated PLC activity by 85 ± 4%, whereas pretreatment with PLC-β_2-, PLC-β_3-, or PLC-β_4-specific antibodies had no significant effect (range of inhibition, 2 ± 7% to 11 ± 10%; not significant) (Fig. 3). Pretreatment with 10 μg/ml $G_{\alpha q/11}$-specific antibody inhibited CCK-stimulated PLC activity by 95 ± 4%, whereas pretreatment with $G_{\alpha i3}$-, $G_{\alpha i1\text{-}2}$-, $G_{\alpha s}$-, or G_β-specific antibodies had no significant effect (range of inhibition, 2 ± 9% to 6 ± 11%; not significant) (Fig. 3). Pretreatment with PTx also had no effect on CCK-stimulated PLC activity (3 ± 7%).

The pattern of inhibition of adenosine-induced contraction and $^{45}Ca^{2+}$ efflux in permeabilized muscle cells by G protein- and PLC-specific antibodies was similar to that obtained for inhibition of adenosine-stimulated PLC activity in membranes. Adenosine-induced contraction in permeabilized muscle cells (9.3 ± 1.3% decrease in cell length) was similar in magnitude to that in intact muscle cells (8 ± 1% decrease in cell length). Blockade of A_2ARs with CGS-15943 increased contraction (25 ± 2% decrease in cell length) and Ca^{2+} efflux (27 ± 1% decrease in steady state cell $^{45}Ca^{2+}$ content) to maximal levels similar to those elicited by 1 μM IP_3 (contraction, 29 ± 2% decrease in cell length; Ca^{2+} efflux, 30 ± 3% decrease in $^{45}Ca^{2+}$ cell content).

Pretreatment of the cells for 1 hr with 10 μg/ml $G_{\alpha i3}$-specific antibody inhibited adenosine-induced contraction and $^{45}Ca^{2+}$ efflux by 97 ± 10% and 61 ± 4%, respectively, whereas pretreatment with 10 μg/ml G_β-specific antibody inhibited contraction and $^{45}Ca^{2+}$ efflux by 95 ± 12% and 86 ± 6%, respectively (Figs. 4 and 5). Pretreatment with 10 μg/ml PLC-β_3-specific antibody inhibited adenosine-induced contraction and $^{45}Ca^{2+}$ efflux by 85 ± 4% and 76 ± 8%, respectively (Figs. 4 and 5). No other G protein- or PLC-specific antibody had any significant effect on contraction or $^{45}Ca^{2+}$ efflux (range of inhibition, 0 to 4 ± 8%; not significant). $^{45}Ca^{2+}$ efflux induced by IP_3 was not affected by pretreatment with $G_{\alpha i3}$- or G_β-specific antibodies (inhibition, 1 ± 6% and 4 ± 6%, respectively; not significant). Conversely, pretreatment with $G_{\alpha q/11}$-specific antibody and PLC-β_1-specific antibody selectively inhibited CCK-induced contraction

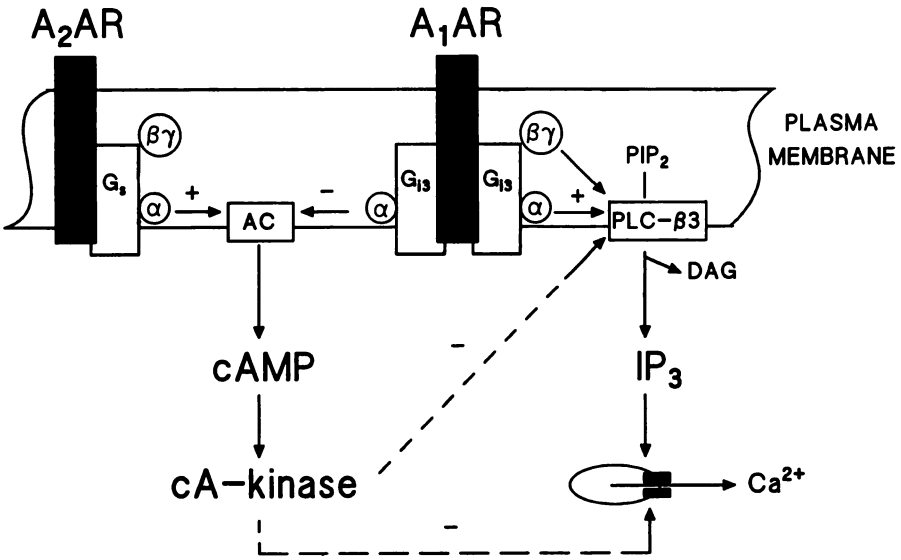

Fig. 7. Model depicting coupling of A_2ARs to activation of adenylyl cyclase (*AC*) via $G_{\alpha s}$ and coupling of A_1ARs to inhibition of adenylyl cyclase via $G_{\alpha i3}$ and activation of PLC-β_3 via both $G_{\alpha i3}$ and $\beta\gamma$. Concurrent activation of adenylyl cyclase by A_2ARs leads to activation of cAMP-dependent protein kinase (*cA-kinase*), which acts to attenuate IP_3 formation and IP_3-dependent Ca^{2+} release. *DAG*, diacylglycerol; *PIP_2*, phosphatidylinositol bisphosphate.

by 95 ± 10% and 81 ± 12%, respectively (Fig. 5); no other G protein- or PLC-specific antibody had any significant effect (range of inhibition, 3 ± 9% to 6 ± 9%; not significant) (Fig. 4).

Identification of G proteins coupled to inhibition and stimulation of adenylyl cyclase by adenosine. G protein-specific antibodies were also used to explore the coupling of A_1ARs and A_2ARs to adenylyl cyclase in permeabilized muscle cells. Pretreatment of the cells with 10 μg/ml $G_{\alpha s}$-specific antibody inhibited adenosine-stimulated cAMP accumulation by 78 ± 4% ($p < 0.001$). The selective A_1AR agonist CPA inhibited forskolin-stimulated cAMP accumulation in permeabilized muscle cells by 75 ± 5% ($p < 0.001$), to the same extent as in intact muscle cells. Pretreatment of the cells with 10 μg/ml $G_{\alpha i3}$-specific antibody reversed the inhibition of forskolin-stimulated cAMP accumulation induced by CPA, whereas pretreatment with 10 μg/ml $G_{\alpha i1}$-, $G_{\alpha i1\text{-}2}$-, $G_{\alpha o}$-, $G_{\alpha q/11}$-, or G_{β}-specific antibodies had no significant effect (Fig. 6).

Discussion

Selective antibodies have been used effectively by several investigators to identify the coupling of G proteins and effector enzymes to cell function (23–25). In the present study, we have used a panel of antibodies selective for PI-specific PLC-β isoforms and various G protein subunits to block adenosine-stimulated PLC activity in muscle membranes and permeabilized dispersed muscle cells from rabbit intestine. Parallel measurements with CCK-8 provided a useful contrast to those obtained with adenosine. Previous studies showed that CCK-stimulated PI hydrolysis in plasma membranes and dispersed muscle cells from guinea pig intestine was insensitive to PTx and mediated by $G_{\alpha q/11}$-dependent activation of PLC-β_1 (17). In contrast, responses mediated by A_1ARs are sensitive to PTx, raising the possibility that they may involve activation of PLC by α or βγ subunits of a PTx-sensitive G protein such as G_o and/or an isoform of G_i (9, 23, 26–32).

Adenosine-stimulated PI-specific PLC activity in dispersed muscle cells and in plasma membranes was sensitive to PTx and the selective A_1AR antagonist DPCPX (30–33), implying that it was mediated by A_1ARs. The receptors were selectively coupled to PLC-β_3, a PLC isoform that is preferentially, although not exclusively, activated by PTx-sensitive G proteins (34). Consistent with this notion, PLC activity was inhibited by both $G_{\alpha i3}$- and G_{β}-specific antibodies. A combination of maximally effective concentrations of both G protein-specific antibodies did not elicit greater inhibition than did either alone, implying that binding of both α and βγ subunits of G_{i3} was required for adenosine-stimulated activation of PLC-β_3. Others have shown that the requirements for activation of effector enzymes by G protein subunits could involve the binding of α and βγ subunits separately or together (23, 35). The dual requirement for activation of PLC-β_3 appears to be unique to adenosine, because activation of PLC-β_3 by somatostatin and δ-opioid receptor agonists is blocked by G_{β}-specific antibody but not by antibodies to the α subunits of G_o or various isoforms of G_i.[1] These distinctive patterns are not consistent with the notion that the effect of the antibodies resulted from blocking of the interaction of the G protein heterotrimer with the receptor. The participation of both $G_{\alpha i3}$ and βγ in the response to adenosine was strongly supported by the results obtained in permeabilized muscle cells, where both contraction and Ca^{2+} release were selectively inhibited by antibodies to $G_{\alpha i3}$, G_{β}, and PLC-β_3.

The pattern of inhibition of CCK-stimulated PLC activity by G protein-specific antibodies was in marked contrast to that observed with adenosine. CCK-stimulated PLC activity in membranes was selectively inhibited by $G_{\alpha q/11}$-specific and PLC-β_1-specific antibodies, consistent with the PTx insensitivity of the response to CCK. Contraction induced by CCK was also selectively inhibited by PLC-β_1- and $G_{\alpha q/11}$-specific antibodies. A similar pattern of selective inhibition of CCK-stimulated PLC activity and contraction by $G_{\alpha q/11}$- and PLC-β_1-specific antibodies was recently observed in dispersed muscle cells and membranes from guinea pig intestine (17). The contrasting results obtained with CCK provide further confirmation of the distinctive pattern of signaling by A_1ARs.

G protein-specific antibodies were also used to identify the coupling of A_1AR and A_2AR to adenylyl cyclase. Stimulation of cAMP accumulation was blocked by $G_{\alpha s}$-specific antibody,

[1] K. S. Murthy and G. M. Makhlouf, unpublished observations.

consistent with A_2AR-dependent activation of G_s. A_1AR-mediated inhibition of cAMP accumulation was demonstrated using the selective A_1AR agonist CPA to inhibit forskolin-stimulated cAMP accumulation (31, 32). The inhibition was selectively reversed by PTx and by $G_{\alpha i3}$-specific antibody, thus confirming the participation of G_{i3} in A_1AR-mediated responses. Only the α subunit of G_{i3} was required for inhibition of adenylyl cyclase, unlike the dual requirement for α and $\beta\gamma$ subunits for activation of PLC-β_3. The effectiveness of the $G_{\alpha i3}$-specific antibody alone in blocking A_1AR-mediated inhibition of adenylyl cyclase and of both $G_{\alpha i3}$- and G_β-specific antibodies in blocking A_1AR-mediated activation of PLC-β_3 is not consistent with the notion that either the α- or β-specific antibody blocked interaction of the G protein heterotrimer with the A_1AR.

A model depicting the coupling of A_1ARs to PLC-β_3 and A_1ARs and A_2ARs to adenylyl cyclase is shown in Fig. 7. The model also depicts the interaction of the signaling pathways. A_2ARs coupled positively to adenylyl cyclase via $G_{\alpha s}$ stimulate cAMP synthesis, whereas A_1ARs coupled negatively to adenylyl cyclase via $G_{\alpha i3}$ attenuate cAMP synthesis. The net effect is stimulation of cAMP accumulation and activation of cAMP-dependent protein kinase. A_1ARs are also coupled via G_{i3} to activation of PLC-β_3 and generation of IP_3 and diacylglycerol. Activation of PLC-β_3 is mediated by the α and $\beta\gamma$ subunits of G_{i3}. The increase in cAMP-dependent kinase activity resulting from activation of A_2ARs attenuates PLC-β_3-dependent generation of IP_3 and diacylglycerol and IP_3-dependent stimulation of Ca^{2+} release (19).

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