0026-895X/94/051036-07$03.00/0

MOLECULAR PHARMACOLOGY, 45:1036-1042

A Single Species of A_1 Adenosine Receptor Expressed in Chinese Hamster Ovary Cells not only Inhibits cAMP Accumulation but also Stimulates Phospholipase C and Arachidonate Release

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Received October 18, 1993; Accepted February 15, 1994

SUMMARY

Chinese hamster ovary cells were transfected with both A_1 adenosine receptor and muscarinic type 3 acetylcholine receptor cDNAs. The muscarinic receptor agonist carbachol stimulated phospholipase C activity, resulting in Ca^{2+} mobilization and arachidonate release. N^6-Cyclopentyladenosine (CPA), an A_1 receptor agonist, did not activate Ca^{2+}-related signal transduction systems by itself but instead inhibited cAMP accumulation. In the presence of carbachol, however, the A_1 receptor agonist enhanced muscarinic receptor agonist-induced phospholipase C/Ca^{2+} responses. In addition, the arachidonate release caused by Ca^{2+} ionophores or thapsigargin was also amplified by CPA, without a change in phospholipase C activity. Thus, CPA augments Ca^{2+}-mediated phospholipase A_2 activation in addition to and separate from its ability to amplify phospholipase C-mediated Ca^{2+} mobilization. Because the permissive actions of CPA on phospholipase C and phospholipase A_2 activation were each reversed by pertussis toxin treatment, in a manner similar to that of the CPA-induced inhibition of cAMP accumulation, we conclude that a single species of A_1 receptor expressed in Chinese hamster ovary cells can couple to multiple signal transduction systems stemming from phospholipase C stimulation, phospholipase A_2-mediated and Ca^{2+}-dependent arachidonate release, and inhibition of cAMP accumulation. A pertussis toxin-sensitive G protein (or proteins) mediates the permissive actions of the A_1 receptor in the stimulation of phospholipase C- and phospholipase A_2-mediated arachidonate release.

Adenosine functions in a variety of physiological processes, including platelet aggregation, smooth muscle vasodilation, neurotransmission, growth and I^- metabolism in the thyroid, lipolysis in adipose tissues, gluconeogenesis and glycogenolysis in the liver, and insulin secretion in β-cells (1–3). Most of the actions of adenosine are mediated by cell surface P_1 purinergic receptors, which in the case of the A_1R and A_2R subtypes couple to adenylate cyclase in an inhibitory and stimulatory manner via PTX-sensitive G_i/G_o and G_s, respectively (4). Adenosine in some cases, however, induces its actions through a cAMP-independent mechanism (1–3).

In FRTL-5 thyroid cells, P_1 agonists inhibit TSH-induced cAMP accumulation, which is accompanied by an inhibition of DNA synthesis (5). Adenosine and its analogues also, however, enhance TSH-induced phospholipase C activation, resulting in Ca^{2+} mobilization and I^- efflux, although the P_1 agonists themselves have no stimulatory effects on the Ca^{2+}-related responses (5). The permissive action of the P_1 agonists on the phospholipase C-Ca^{2+} system was also observed in association with α_1-adrenergic agonists or P_2-purinergic agonists. Thus, P_1 agonists stimulated phospholipase C in FRTL-5 cells previously or simultaneously treated with norepinephrine or P_2 agonists (6–8). The phospholipase C activation in these latter cases was accompanied by arachidonic acid release and the production of lysophosphatidylcholine, suggesting the involvement of phospholipase A_2 activity (9). P_1 agonists also, however, enhanced phospholipase A_2 activation induced by Ca^{2+} ionophores and thapsigargin in a PTX-sensitive manner without any change in phospholipase C activity (9), suggesting that a P_1 receptor-G_i/G_o system directly couples to phospholipase A_2, independently of phospholipase C. The actions of the P_1 agonists in all of these cases did not appear to be a secondary response to changes in cAMP metabolism (6, 7, 9). Taken together, therefore the results suggested that P_1 receptors in FRTL-5 cells are coupled to stimulation of phospholipase C and phospholipase A_2, independently of cAMP changes, as well as to the inhibition

This work was supported in part by a research grant from the Ministry of Education, Science, and Culture of Japan. M.A. is a Monbusho Scholar.

ABBREVIATIONS: PTX, pertussis toxin; A_1R and A_2R, A_1 and A_2 types of adenosine (P_1) receptor(s); M_3R, muscarinic type 3 acetylcholine receptor(s); $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; RO 20–1724, 4-(3-butoxy-4-methoxybenzoyl)-2-imidazolidinone; CPA, N^6-cyclopentyladenosine; PIA, N^6-(L-2-phenylisopropyl)adenosine; CADO, 2-chloroadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; QNB, quinuclidinyl benzilate; TSH, thyrotropin; SRα, pCDL-SRα; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SV40, simian virus 40.

of adenylate cyclase. Similar stimulation of cAMP-independent signal transduction systems by P_1 agonists has been reported in other cell types (3, 10–13). These P_1 agonist actions appear to be mediated by an A_1 type of receptor, based on the PTX sensitivity and pharmacological specificity of agonist and antagonist actions (8); however, the possibility cannot be ruled out that receptor subtypes different from the A_1R for adenylate cyclase inhibition mediate the cAMP-independent adenosine actions (14).

In CHO cells transfected with muscarinic acetylcholine receptor cDNA, muscarinic receptor agonists activate phospholipase C (15). When CHO cells are transfected with dopamine D_2 receptor cDNA, D_2 receptor agonists inhibit adenylate cyclase activity and potentiate Ca^{2+} ionophore-induced phospholipase A_2-mediated arachidonate release (16). Both D_2 receptor responses are reversed by PTX treatment, suggesting the involvement of G_i/G_o in phospholipase A_2 activation as well as adenylate cyclase inhibition. This is similar to the case of P_1 agonist actions in FRTL-5 cells (6, 9). The results thus indicate that CHO cells have cAMP-independent phospholipase C and A_2 systems, as well as an inhibitory adenylate cyclase system, which can couple to exogenous receptors expressed in the cells by gene transfection.

Recently, cDNA encoding A_1R has been identified in many tissues and species (17–20). To clarify whether a single species of thyroid A_1R couples to the cAMP-independent signal transduction systems as well as to adenylate cyclase inhibition, CHO cells were transfected with A_1R cDNA derived from dog thyroid cells. We show that A_1R agonists not only inhibit cAMP accumulation but also enhance the Ca^{2+}-induced phospholipase A_2 activation. When M_3R cDNA is cotransfected with A_1R cDNA, we additionally show that the ability of the muscarinic receptor agonist carbachol to induce phospholipase C and A_2 activation is enhanced by A_1R agonists. These data indicate that a single species of thyroid A_1R can couple to multiple signal transduction systems.

Experimental Procedures

Materials. PIA, CADO, forskolin, thapsigargin, A23187, and carbachol were purchased from Sigma Chemical Co.; CPA and DPCPX were from Research Biochemicals Inc., ionomycin from Calbiochem, and L-[^{3}H]QNB (52.3 Ci/mmol) and [^{3}H]DPCPX (109.2 Ci/mmol) from New England Nuclear. PTX was kindly provided by Dr. M. Ui (Tokyo University), SRα296 vector (21) by Dr. Y. Takebe (National Institute of Health, Japan), dog A_1R cDNA (17) (inserted into pBluescript) by Dr. G. Vassart (Universite Libre de Bruxelles), and porcine M_3R cDNA (inserted into SV40 vector; SV40M_3R) (22, 23) by Dr. S. Numa (Kyoto University). Radioimmunoassay of cAMP used a Yamasa cAMP assay kit, which was the kind gift from Yamasa Shoyu Co. (Choshi, Chiba). The sources of all other reagents were as described in previous papers (5–9, 24–26).

Cell culture and cDNA transfection. CHO cells were cultured in 10-cm dishes, in Ham's F-12 medium supplemented with 10% heat-inactivated fetal calf serum, in a 5% CO_2 atmosphere at 37°. The cells were harvested with 0.05% trypsin and 0.53 mM EDTA (GIBCO) for transfection experiments.

The full length cDNA for dog A_1R was released from pBluescript vector (Stratagene, La Jolla, CA) by *Eco*RI digestion, blunt-ended using a blunt-ending kit (Takara, Kyoto, Japan), and subcloned into the *Sma*I site of pGEM3Z vector (Promega, Madison, WI). The cDNA insert was then excised with *Pst*I and *Eco*RI and subcloned into the *Pst*I/*Eco*RI sites of SRα to produce a SRαA_1R expression vector for transfection experiments. The SRα, SRαA_1R, SV40, and SV40M_3R vectors were transfected into CHO cells (20 μg of DNA in 0.8 ml) by electroporation (0.3 kV, 500 μF) and were subcultured into 24-well plates for arachidonate release, cAMP measurement, inositol phosphate assay, and [^{3}H]DPCPX binding experiments and into 10-cm dishes for Ca^{2+} and [^{3}H]QNB binding experiments. For inositol phosphate assays, inositol-free Dulbecco's modified Eagle's medium supplemented with [^{3}H]inositol (1 μCi/ml) and 10% fetal calf serum was used instead of Ham's F-12 medium supplemented with 10% fetal calf serum. The cells were cultured for 2 days before the following assays were performed.

Inositol phosphate assay. The cells cultured with [^{3}H]inositol were washed twice with HEPES-buffered medium composed of 10 mM HEPES, pH 7.5, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V) and were then incubated for 1 hr at 37° with the same medium containing 10 mM LiCl and the agents to be tested, in a final volume of 0.5 ml. The reaction was terminated by aspiration of the medium and addition of 0.6 ml of 0.1 N HCl. [^{3}H] Inositol phosphates, including inositol mono-, di-, and triphosphates, were separated from inositol and glycerophosphoinositol on Dowex 1X8 formate columns, as described previously (6).

$[Ca^{2+}]_i$ measurement. The cells were harvested from 10-cm dishes with phosphate-buffered saline containing 4 mM EDTA. $[Ca^{2+}]_i$ was estimated from the change in the fluorescence of fura-2-loaded cells, as described previously (6, 26).

Arachidonate release. Cells labeled for 6 hr with [^{3}H]arachidonate were washed three times, at 5-min intervals, with HEPES-buffered medium. Cells were then incubated at 37° with the test agents, in a final volume of 0.75 ml of the same medium. Ten minutes later, the medium (0.5 ml) was removed and counted in a liquid scintillation counter (Beckman LS 7500).

Measurement of cAMP content. The cells were washed twice with HEPES-buffered medium and then incubated for 10 min in the same medium, with the agents to be tested, in the presence of 0.1 mM RO 20–1724 and 50 μM forskolin unless otherwise stated. Termination of the reaction and measurement of cAMP content were performed as described previously (6).

Radioligand binding assays. For the [^{3}H]QNB binding assay, crude membranes were prepared by essentially the same methods as described previously (6). Approximately 50 μg of membranes were incubated for 30 min at 25° with increasing doses (0.078–10 nM) of [^{3}H]QNB, a muscarinic antagonist, in the absence or presence of atropine (10 μM). The incubation medium was 50 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EGTA, and 0.05% bovine serum albumin. The reaction was stopped by the addition of 3 ml of ice-cold Tris·HCl buffer containing 5 mM $MgCl_2$ and 1 mM EGTA. The reaction mixture was immediately filtered, under vacuum, through a Whatman GF/B glass fiber filter (24-mm diameter). The filter was rapidly washed twice with the same buffer (3 ml) and dried at 80° for 30 min before being counted for radioactivity. Specific binding was defined as the radioactivity displaceable by 10 μM atropine.

For the [^{3}H]DPCPX binding assay, the cells were washed twice with HEPES-buffered medium and then incubated for 30 min with [^{3}H] DPCPX at 25°. Incubation was terminated by aspiration of the medium and washing of the cells twice with ice-cold HEPES-buffered medium. Radioactive ligand bound to the cells were extracted with 5% trichloroacetic acid, and the radioactivity was measured in a liquid scintillation counter. Specific binding was defined as the radioactivity displaceable by the addition of 1 μM unlabeled DPCPX.

Data presentation. All experiments were performed in triplicate. The results of multiple observations are presented as the mean value of results from more than three different batches of cells, unless otherwise stated.

Results

Enhancement by the A_1R agonist CPA of carbachol-induced phospholipase C activation and Ca^{2+} mobilization in CHO cells cotransfected with A_1R and M_3R cDNAs. In CHO cells transfected with SRα plus SV40 vectors (Fig. 1A) or with A_1R plus SV40 vectors (Fig. 1B), no detectable

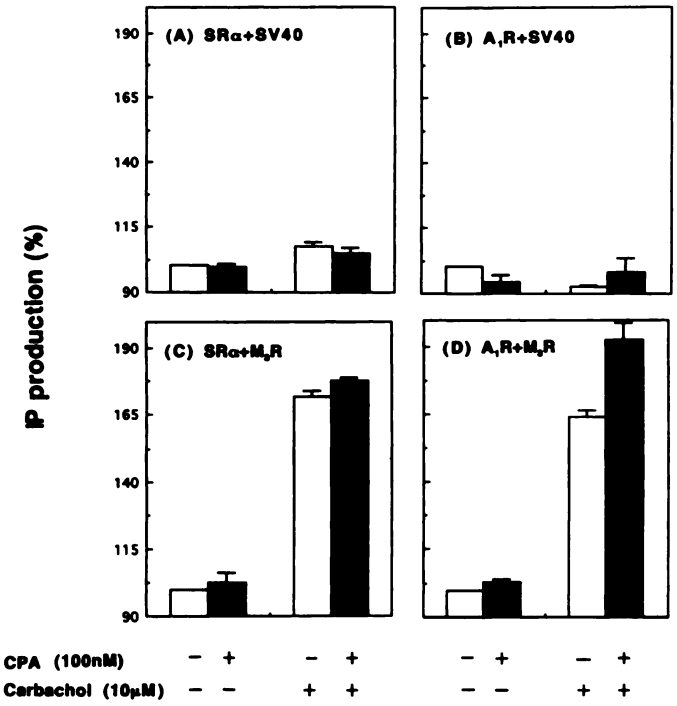

Fig. 1. Effect of carbachol and CPA on production of inositol phosphates (*IP*), including inositol mono-, di-, and triphosphates, in control (SRα and SV40 vector-transfected) (A), A_1R-expressing (A_1R and SV40 vector-transfected) (B), M_3R-expressing (SRα and M_3R vector-transfected) (C), and both A_1R- and M_3R-expressing (A_1R and M_3R vector-transfected) (D) CHO cells. The cells were incubated with 10 μM carbachol in the absence or presence of 100 nM CPA. Data are expressed as percentages of the basal (without any agonist) values, taken as 100%.

activation of inositol phosphate production by carbachol, CPA, or the combination of these agonists was observed. In cells transfected with SRα plus M_3R vectors, inositol phosphate production, reflecting phospholipase C activity, was increased in response to carbachol, but CPA did not affect the enzyme activity either in the presence or in the absence of the muscarinic agonist (Fig. 1C). In cells cotransfected with A_1R and M_3R, although CPA alone had no stimulatory action on the enzyme activity, the A_1R agonist enhanced the carbachol-induced activation (Fig. 1D). The increment, although small, was very consistent in multiple experiments, with statistically significant effect ($p < 0.01$). Thus, the A_1R agonist permissively activates phospholipase C.

The number of A_1R expressed in these cells was estimated by measuring the binding of the A_1R-specific antagonist [^{3}H]DPCPX to intact CHO cells, because the membrane fractions did not show saturable binding of [^{3}H]DPCPX at concentrations up to 40 nM. We observed specific [^{3}H]DPCPX binding to cells transfected with A_1R and SV40 vectors but not to control cells transfected with either SRα and SV40 vectors or SRα and M_3R vectors (data not shown). Scatchard analysis of the binding data revealed a single class of [^{3}H]DPCPX binding sites, with a dissociation constant (K_d) of 11.4 ± 4.1 nM and a maximal binding capacity (B_{max}) of 1.05 ± 0.26 pmol/mg of cell protein (three experiments). This B_{max} value is not far from that obtained with FRTL-5 thyroid cells, in which A_1R are expressed natively, although a different A_1R antagonist, [^{3}H] 1,3-diethyl-8-phenylxanthine, was used for the binding assay (6).

Cotransfection of A_1R with M_3R did not essentially affect the parameters of [^{3}H]DPCPX binding to the cells; the K_d and B_{max} values were 17.5 ± 1.5 nM and 0.67 ± 0.04 pmol/mg of cell protein, respectively (three experiments).

To analyze M_3R expression, we measured [^{3}H]QNB binding to the membrane fraction of CHO cells. Specific [^{3}H]QNB binding to the membranes was detected only in cells transfected with the M_3R vector. When the cells were transfected with both A_1R and M_3R vectors, Scatchard analysis of the binding data revealed the presence of a single class of [^{3}H]QNB binding sites, with a K_d of 0.25 nM and a B_{max} of 176 fmol/mg of membrane protein (two experiments). This B_{max} value is within the range of reported values for native M_3R in membrane preparations (27, 28). Thus, both A_1R and M_3R were expressed on the CHO cell membranes within their physiological ranges.

Fig. 2 shows a change in $[Ca^{2+}]_i$ in CHO cells expressing A_1R and M_3R. In the presence of 2 mM extracellular Ca^{2+}, carbachol alone increased $[Ca^{2+}]_i$, but CPA alone did not (Fig. 2, A and D). In the presence of carbachol, however, CPA clearly increased $[Ca^{2+}]_i$, and the carbachol-induced $[Ca^{2+}]_i$ rise was higher in cells that had been previously treated with CPA than in control cells (Fig. 2, A and D). Similar cross-talk between CPA and carbachol was observed under conditions of low extracellular Ca^{2+} concentrations, wherein an excess amount of EGTA was added to the incubation medium; CPA increased $[Ca^{2+}]_i$ in the presence of carbachol but not in its absence (Fig. 2, B and E).

The effect of PTX on the CPA- and carbachol-induced $[Ca^{2+}]_i$ rise was examined in Fig. 2, C and F. The permissive action of CPA was completely reversed by PTX treatment of the cells, whereas the carbachol effect was unchanged under these conditions, indicating the involvement of PTX-sensitive G proteins in the CPA-induced but not the carbachol-induced Ca^{2+} mobilization.

Effect of CPA on arachidonate release. Carbachol but not CPA could also induce arachidonate release in cells transfected with SRα plus M_3R vectors (Fig. 3C), but the muscarinic receptor agonist, the A_1R agonist, or the combination of these agonists did not induce significant release in the cells transfected with SRα plus SV40 vectors (Fig. 3A) or with A_1R plus SV40 vectors (Fig. 3B). In cells cotransfected with A_1R and M_3R, CPA did not have any effect when added alone but enhanced the carbachol-induced arachidonate release (Fig. 3D).

The dose-dependent effect of CPA on the arachidonate release in cells expressing both A_1R and M_3R is shown in Fig. 4. CPA alone had no stimulatory effect on the activity at up to 1 μM but enhanced the carbachol-induced response with a half-maximal effective dose of approximately 30 nM (Fig. 4A). PTX treatment completely abolished the CPA action (Fig. 4B).

In CHO cells, receptor-mediated arachidonate release appears to be mediated by a Ca^{2+}-dependent activation of phospholipase A_2 (16). Because CPA enhanced the carbachol-induced phospholipase C activity and the subsequent $[Ca^{2+}]_i$ increase (Figs. 1 and 2), the CPA enhancement of carbachol-induced arachidonate release may be explained by an amplification of the Ca^{2+} response. Thapsigargin, an inhibitor of endoplasmic reticulum ATPase, and Ca^{2+} ionophores, including A23187 and ionomycin, can also induce arachidonate release (Fig. 5). As shown in Fig. 5, CPA enhanced the actions of all of the Ca^{2+} mobilizers to increase arachidonate release. Under these conditions, no detectable increase in the activity of phospholipase C was observed (data not shown). This suggests that A_1R can couple to the phospholipase A_2 system directly, if

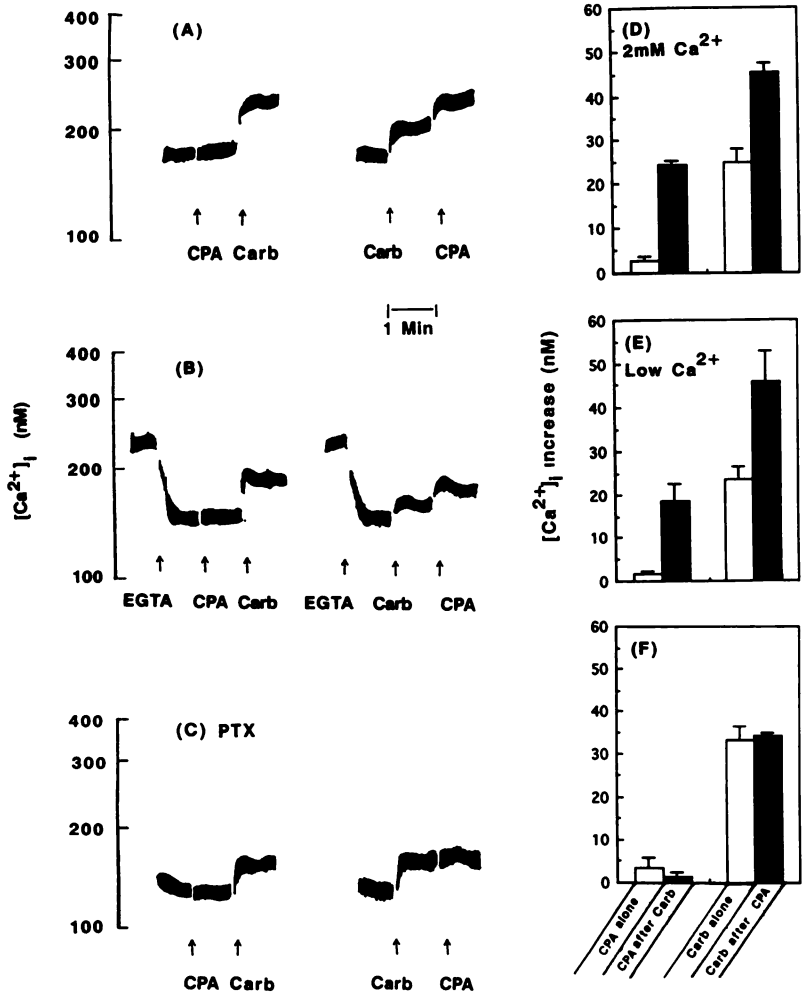

Fig. 2. Change in $[Ca^{2+}]_i$ produced by CPA and carbachol (*Carb*) in both A_1R- and M_3R-expressing CHO cells. The cells were treated (C and F) or not treated (A, B, D, and E) with PTX (100 ng/ml) for 18 hr. Five minutes before the start of monitoring of the fluorescence change, apyrase (1 unit/ml) was added in the incubation medium. Representative traces of the $[Ca^{2+}]_i$ changes produced by CPA (100 nM), carbachol (500 nM), and EGTA (3 mM) are shown in A, B, and C. The peak rises in $[Ca^{2+}]_i$ induced by CPA or carbachol under the conditions noted are summarized in D, E, and F.

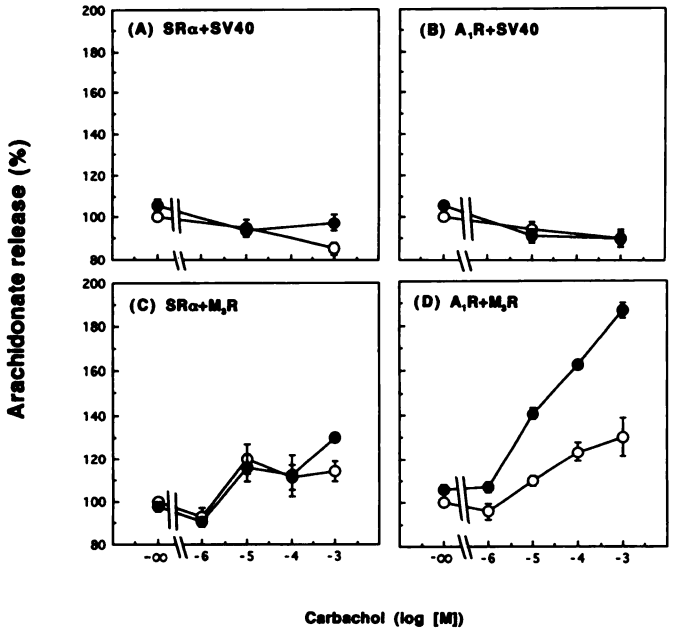

Fig. 3. Dose-dependent effect of carbachol on arachidonate release in control cells (transfected with SRα and SV40 vectors) (A) and in cells expressing A_1R alone (B), M_3R alone (C), or both A_1R and M_3R (D). The cells were incubated with the indicated doses of carbachol in the presence (●) or absence (○) of 100 nM CPA. Data are expressed as percentages of the basal (without any agonist) values, taken as 100%.

$[Ca^{2+}]_i$ attains a certain level, in addition to activation of the phospholipase C.

Inhibition of cAMP accumulation by A_1R agonists. Consistent with previous observations (17), CPA inhibited cAMP accumulation in a dose-dependent fashion in CHO cells expressing A_1R (Fig. 6B). The CPA action was again almost completely reversed by PTX treatment (Fig. 6B).

The potencies of A_1R agonists to inhibit cAMP accumulation (Fig. 6B) were compared with those to stimulate arachidonate release (Fig. 6A). Half-maximally effective doses of CPA, PIA, and CADO were the same in each assay and were within the range of 10–30 nM.

Evidence that a decrease in cAMP accumulation is not responsible for the CPA-induced enhancement of arachidonate release. The foregoing results indicate that the expressed A_1R can couple to or modulate at least three effector enzymes, i.e., phospholipase C (stimulation), phospholipase A_2 (activation), and adenylate cyclase (inhibition). To rule out the possibility that the phospholipase C and phospholipase A_2 activation by A_1R agonists is secondary to adenylate cyclase inhibition, we compared the A_1R agonist actions in the presence and absence of forskolin, an adenylate cyclase-activating agent. In cells without forskolin treatment, CPA caused significant changes in the cAMP content (Table 1). The cAMP content was increased about 1000-fold by the additin of 50 μM forskolin and even in the presence of 100 nM CPA was at at least 500 times higher than the levels in the absence of forskolin. The

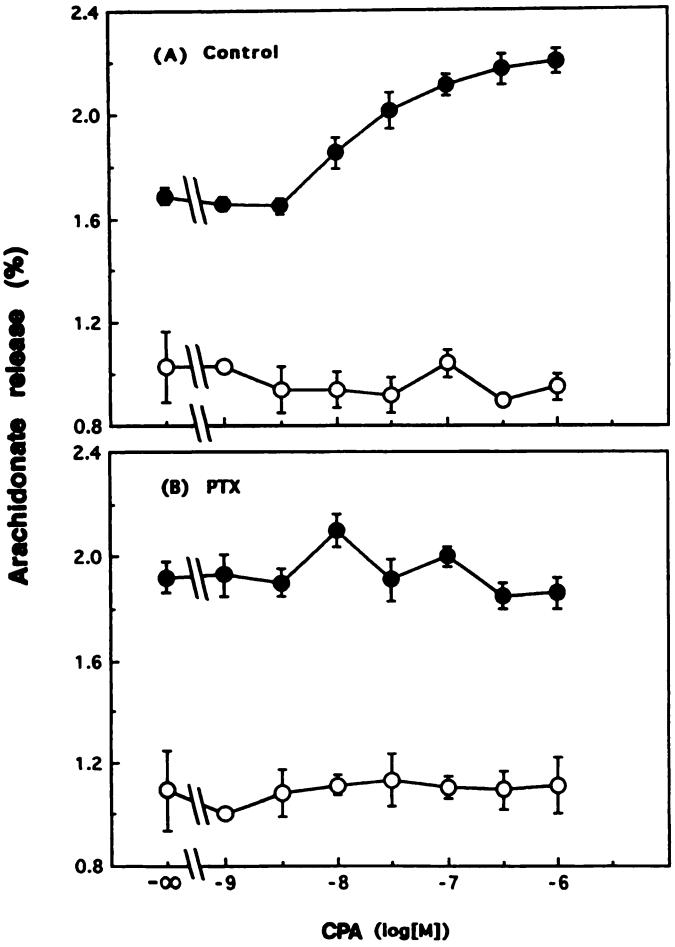

Fig. 4. Dose-dependent effect of CPA on arachidonate release in cells expressing both A_1R and M_3R. Cells that had been pretreated with (B) or without (A) PTX (100 ng/ml) for 18 hr were incubated with the indicated doses of CPA in the presence (●) or absence (○) of 10 μM carbachol. Data are expressed as percentages of total radioactivity incorporated into the cells.

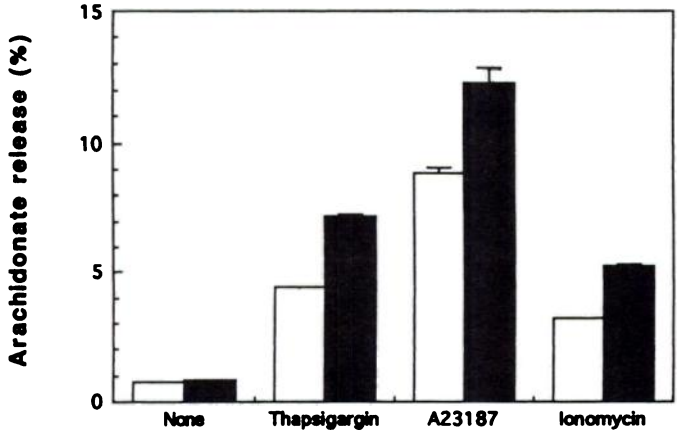

Fig. 5. Effect of CPA (100 nM) on thapsigargin (1 μM)-, A23187 (1 μM)-, and ionomycin (1 μM)-induced arachidonate release in cells expressing A_1R. Data are expressed as percentages of total radioactivity incorporated into the cells. □, Without CPA; ■, with CPA.

CPA action to enhance carbachol-induced arachidonate release was not significantly influenced by such remarkable elevation of cAMP levels. The results indicate that the decrease in cAMP content cannot account for the CPA enhancement of phospholipase A_2 activity. Dissociation of the decrease in cAMP levels

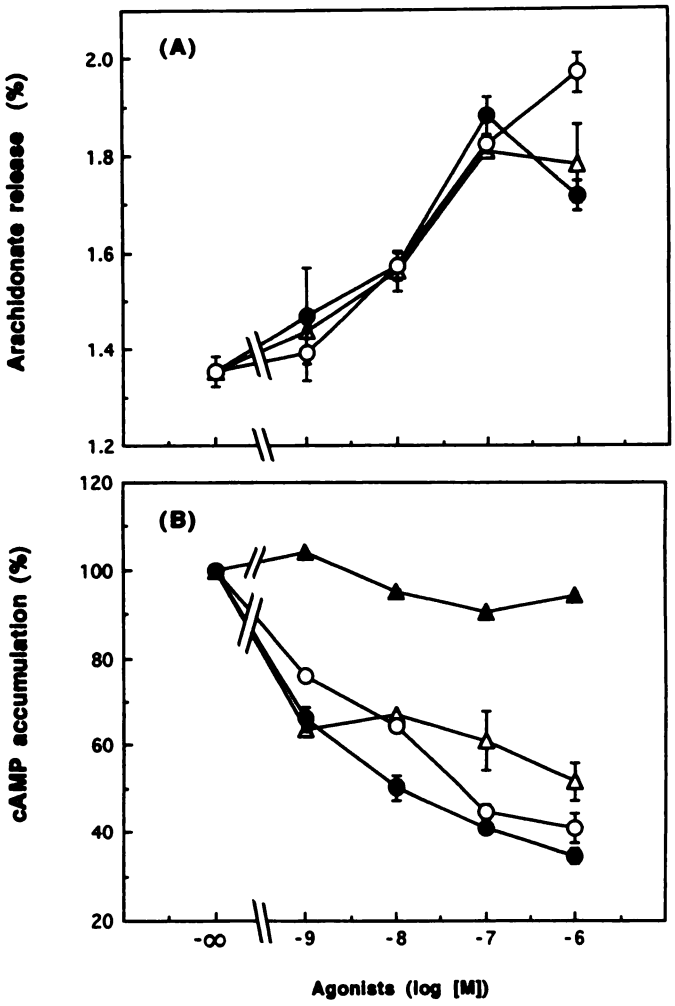

Fig. 6. Dose-dependent effect of A_1R agonists on arachidonate release (A) and cAMP accumulation (B) in cells expressing both A_1R and M_3R. The cells were incubated with 10 μM carbachol (A) and with 50 μM forskolin and 0.1 mM RO 20–1724 (B), together with the indicated dose of CPA (○), PIA (△), or CADO (●). In B, the effect of PTX (which was added in the culture medium 18 hr before the experiment) on the CPA dose-response curve was also assayed (▲). Data are expressed as percentages of total radioactivity incorporated into the cells in A and as percentages of values in the absence of A_1R agonists in B.

TABLE 1

Effect of forskolin on CPA enhancement of carbachol-induced arachidonate release

Cells expressing both A_1R and M_3R were incubated with 10 μM carbachol and/or 100 nM CPA in the absence (control) or presence of 50 μM forskolin for 10 min, and then cAMP content was measured. For arachidonate release, experimental conditions were the same as those for the cAMP experiment, except that the cells had been incubated with [^{3}H]arachidonic acid. Data are expressed as percentages of basal (without any agonist) values, taken as 100% for arachidonate release. Forskolin treatment did not significantly change the basal activity of arachidonate release.

	Arachidonate release		cAMP content	
	Control	Forskolin	Control	Forskolin
	%		*pmol/well*	
Carbachol	130 ± 6	124 ± 4	1.6 ± 0.1	1540 ± 83
Carbachol + CPA	164 ± 3[a]	166 ± 10[a]	1.2 ± 0.1[b]	860 ± 44[a]

[a] CPA effect is significant, $p < 0.01$.
[b] $p < 0.05$.

produced by CPA from the permissive action of CPA on carbachol actions was also seen for the carbachol-induced $[Ca^{2+}]_i$ increase (data not shown).

Discussion

In the present paper, we show that adenosine analogues inhibit adenylate cyclase activation, resulting in a decrease of cAMP accumulation in CHO cells expressing A_1R and M_3R. We show also that adenosine analogues enhance carbachol-induced phospholipase C activation and associated $[Ca^{2+}]_i$ increases, as well as phospholipase A_2 activation induced by ionophores or thapsigargin in the absence of phospholipase C activation. This is, to our knowledge, the first indication that a single species of A_1R can stimulate cAMP-independent signal transduction systems as well as the inhibitory adenylate cyclase system.

A similar permissive action of adenosine and its analogues on phospholipase C activation, followed by Ca^{2+} mobilization, has been shown in FRTL-5 thyroid cells. Thus, P_1 agonists enhanced the phospholipase C activation and $[Ca^{2+}]_i$ increase induced by P_2 purinergic agonists (6, 8, 9), α_1-adrenergic agonists (7), or TSH (5) in a PTX-sensitive manner. Adenosine and its analogues have also been reported to enhance the actions of P_2 agonists and bradykinin in smooth muscle cells (12, 13) and of IgE in RBL 2H3 basophils (10), although in these latter cases the effects of the P_1 receptor agonists were synergistic and not permissive. Thus, the agonists themselves slightly but significantly stimulated Ca^{2+} mobilization. In this study and in this last regard, we noticed that CPA induced a small but significant $[Ca^{2+}]_i$ increase in the A_1R-expressing CHO cell suspensions when apyrase, a mixture of ATPase and ADPase, was omitted from the medium (data not shown). This result is in agreement with a recent report on spontaneous ATP release from CHO cells (29) and suggests that the released ATP or ADP, Ca^{2+}-mobilizing agonists, might sensitize the cells. The permissive effect of an A_1R agonist added externally thus becomes recognizable only if apyrase is added. In the case of phospholipase C activity measured using unharvested cells, however, the addition of CPA alone did not induce a positive response even in the absence of apyrase (Fig. 1). It is thus reasonable to assume a higher sensitivity of the Ca^{2+} response than of the inositol phosphate response and/or a higher releasing rate for adenine nucleotides in cell suspensions than in unharvested cells. These observations in the A_1R-expressing CHO cells may be applicable to understanding the apparent synergistic activity of A_1R agonists to induce Ca^{2+} mobilization in smooth muscle cells and RBL 2H3 cells, i.e., the presence of autocrine ATP or ADP plays a synergistic role in these cells. The permissive effects in our system would not, therefore, conflict with the synergism seen in smooth muscle cells and RBL 2H3 cells.

In the present experimental system, we did not determine whether both A_1R and M_3R were expressed in the same single cells. Therefore, the observed permissive effect of CPA on the carbachol action might reflect intercellular, but not intracellular, events between the A_1R- and M_3R-expressing cells, mediated by putative paracrine factors that are produced by CPA or carbachol. Similar cross-talk events, however, have been observed in cell lines where two types of receptors are constitutively expressed, such as FRTL-5 cells (5–9), a cell line derived from smooth muscle (12, 13), and RBL 2H3 cells (10). Furthermore, our preliminary experiments using CHO cell lines stably expressing the recombinant A_1R showed similar permissive effects of CPA on Ca^{2+} mobilization induced by UTP, an agonist for the endogenous P_{2U} receptor. These facts support our concept that the present observations are based on mechanisms of cross-talk between A_1R and M_3R systems expressed in the same single cells, although the possible involvement of paracrine actions, as mentioned above, may not be completely excluded.

The CPA stimulation of phospholipase C was PTX sensitive, whereas the carbachol effect on the enzyme was insensitive to the toxin. This suggests the involvement of G_i/G_o in the permissive action of the A_1R agonist on muscarinic induction of phospholipase C but not in the muscarinic action itself. It has been widely recognized that there are two types of G proteins involved in phospholipase C activation systems (30, 31). Recently, the G_q family of G proteins have been identified as the PTX-insensitive G proteins in the system (32), whereas G_i/G_o [it is not yet clear which subtype(s)] is PTX sensitive. Very recently, the $\beta\gamma$ subunits of G_i/G_o have been shown to activate the $\beta 2$ type of phospholipase C (33, 34). Because PTX inhibits the receptor-mediated dissociation of G_i/G_o composed of $\alpha\beta\gamma$ to α and $\beta\gamma$ (35), the sensitivity of the phospholipase C system to PTX may be explained by toxin inhibition of $\beta\gamma$ production, which is responsible for the activation of the enzyme. This mechanism is, however, not applicable to the permissive action of A_1R agonists, because the mechanism should work when the A_1R agonists are added alone. On the other hand, Harden and colleagues (36) have shown that, in their turkey erythrocyte system, the $\beta\gamma$ complexes of G proteins enhance the P_2 receptor-mediated activation of the enzyme (probably $\beta 1$ type). In this regard, $\beta\gamma$ has been suggested to bind with an inactive (GDP-bound) α subunit of G_q or one of the PTX-insensitive G proteins. This may facilitate G protein coupling to Ca^{2+}-mobilizing receptors by accelerating the recovery of the heterotrimeric form (36, 37).

CPA enhancement of the carbachol-activated phospholipase C-Ca^{2+} system can increase arachidonate release, because phospholipase A_2 is a Ca^{2+}-dependent enzyme and because the CPA effect amplifies Ca^{2+} mobilization by carbachol. This CPA action was reversed by PTX treatment. Thapsigargin- or Ca^{2+} ionophore-induced $[Ca^{2+}]_i$ increases, resulting in arachidonate release, are phospholipase C independent; under these experimental conditions, both effects were, unexpectedly, further enhanced by CPA. This action of CPA was also PTX sensitive. A similar enhancement of the Ca^{2+}-mediated arachidonate release has been reported in dopamine D_2 receptor-expressing CHO cells, where dopamine in the presence of A23187 induced arachidonate release (16) in a PTX-sensitive manner. These results suggest that either expressed A_1R or D_2 receptors can couple directly to a phospholipase A_2 system via G_i/G_o, provided that $[Ca^{2+}]_i$ attains a certain threshold level. Thus, enhancement by the A_1R agonist of carbachol-induced arachidonate release may be due to enhancement of Ca^{2+}-mediated phospholipase A_2 activation in addition to amplification of phospholipase C-induced Ca^{2+} mobilization. The role of Ca^{2+} in phospholipase A_2 activation in CHO cells is still unclear, but recent studies suggest that an increase in $[Ca^{2+}]_i$ induces the translocation of phospholipase A_2 from cytosol to plasma membranes (38–40). It is therefore reasonable to assume that an increase in the enzyme bound to the membranes facilitates the coupling of the enzyme to A_1R- or D_2 receptor-G_i/G_o systems. The up-regulation of phospholipase A_2 by the $\beta\gamma$ complexes of G

proteins has been reported in rod outer segments of the retina (41).

We demonstrate in this report that a single species of A_1R can couple to cAMP-independent signal transduction pathways, as well as an inhibitory adenylate cyclase pathway, via PTX-sensitive G protein(s). Such a permissive role of G_i/G_o is not restricted to the case where the G proteins are stimulated by A_1R. In NG108–15 neuroblastoma-glioma hybrid cells, inhibitory agonists for adenylate cyclase, such as somatostatin, enkephalin, and α_2-adrenergic agonists, also stimulate the phospholipase C-Ca^{2+} system in a PTX-sensitive manner, if the cells have been sensitized by a Ca^{2+}-mobilizing agonist, e.g., P_2 agonists or bradykinin (24–26). Thus, an inhibitory signal for adenylate cyclase may generally be associated with an ability to modulate cAMP-independent phospholipase C and A_2 activity. Whether a single species of G_i/G_o mediates all of the PTX-sensitive responses mediated by A_1R, as predicted by this association, or whether each response is mediated by a different species of G_i/G_o is under investigation.

References

1. Daly, J. W. Role of ATP and adenosine receptors in physiologic processes: summary and prospects, in *Physiology and Pharmacology of Adenosine* (J. W. Daly, Y. Kuroda, J. W. Phillis, H. Shimizu, and M. Ui, eds.). Raven Press, New York, 275–290 (1983).
2. Olsson, R. A., and J. D. Pearson. Cardiovascular purinoceptors. *Physiol. Rev.* **70**:761–849 (1990).
3. Fredholm, B. B., and T. V. Dunwiddie. How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci.* **9**:130–134 (1988).
4. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77**:2551–2554 (1980).
5. Sho, K., F. Okajima, M. A. Majid, and Y. Kondo. Reciprocal modulation of thyrotropin actions by P_1-purinergic agonists in FRTL-5 thyroid cells. *J. Biol. Chem.* **265**:12180–12184 (1991).
6. Okajima, F., K. Sato, M. Nazarea, K. Sho, and Y. Kondo. A permissive role of pertussis toxin substrate G-protein in P_2-purinergic stimulation of phosphoinositide turnover and arachidonate release in FRTL-5 thyroid cells. *J. Biol. Chem.* **264**:13029–13037 (1989).
7. Okajima, F., K. Sato, K. Sho, and Y. Kondo. Stimulation of adenosine receptor enhances α_1-adrenergic receptor-mediated activation of phospholipase C and Ca^{2+} mobilization in a pertussis toxin-sensitive manner in FRTL-5 thyroid cells. *FEBS Lett.* **248**:145–149 (1989).
8. Nazarea, M., F. Okajima, and Y. Kondo. P_2-purinergic activation of phosphoinositide turnover is potentiated by A_1-receptor stimulation in thyroid cells. *Eur. J. Pharmacol.* **206**:47–52 (1991).
9. Shimegi, S., F. Okajima, and Y. Kondo. Permissive stimulation of Ca^{2+}-induced phospholipase A_2 by an adenosine receptor agonist in a pertussis toxin-sensitive manner in FRTL-5 thyroid cells. *Biochem. J.*, **299**:845–851 (1994).
10. Ali, H., J. R. Cunha-Melo, W. F. Saul, and M. A. Beaven. Activation of phospholipase C via adenosine receptors provides synergistic signal for secretion in antigen-stimulated RBL-2H3 cells. *J. Biol. Chem.* **265**:745–753 (1990).
11. Olivera, A., A. Lopez-Rivas, and J. M. Lopez-Novoa. Adenosine stimulates Ca^{2+} fluxes and increases cytosolic free Ca^{2+} in cultured rat mesangial cells. *Biochem. J.* **282**:871–876 (1992).
12. Gerwins, P., and B. B. Fredholm. Stimulation of adenosine A_1 receptors and bradykinin receptors, which act via different G proteins, synergistically raises inositol 1,4,5-trisphosphate and intracellular free calcium in DDT_1MF-2 smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **89**:7330–7334 (1992).
13. Gerwins, P., and B. B. Fredholm. ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. *J. Biol. Chem.* **267**:16081–16087 (1992).
14. Ribeiro, J. A., and A. M. Sebastiao. Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.* **26**:179–209 (1986).
15. Ashkenazi, A., E. G. Peralta, J. W. Winslow, J. Ramachandran, and D. J. Capon. Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* **56**:487–493 (1989).
16. Piomelli, D., C. Pilon, B. Giros, P. Sokoloff, M. P. Martres, and J. C. Schwartz. Dopamine activation of the arachidonic acid cascade as a basis for D_1/D_2 receptor synergism. *Nature (Lond.)* **353**:164–167 (1991).
17. Libert, F., S. N. Schiffmann, A. Lefort, M. Parmentier, C. Gerard, J. E. Dumont, J.-J. Vanderhaeghen, and G. Vassart. The orphan receptor cDNA RDC7 encodes an A_1 adenosine receptor. *EMBO J.* **10**:1677–1682 (1991).
18. Mahan, L. C., L. D. Mcvittie, E. M. Smyk-Randall, H. Nakata, F. J. Monsma, Jr., C. R. Gerfen, and D. R. Sibley. Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**:1–7 (1991).
19. Reppert, S. M., D. R. Weaver, J. H. Stehle, and S. A. Rivkees. Molecular cloning and characterization of a rat A_1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.* **5**:1037–1048 (1991).
20. Tucker, A. L., J. Linden, A. S. Robeva, D. D. D'Angelo, and K. R. Lynch. Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett.* **297**:107–111 (1992).
21. Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokata, K. Arai, M. Yoshida, and N. Arai. SRα promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**:466–472 (1988).
22. Akiba, I., T. Kubo, A. Maeda, H. Bujo, J. Nakai, M. Mishina, and S. Numa. Primary structure of porcine muscarinic acetylcholine receptor III and antagonist binding studies. *FEBS Lett.* **235**:257–264 (1988).
23. Nukada, T., M. Mishina, and S. Numa. Functional expression of cloned cDNA encoding the α-subunit of adenylate cyclase-stimulating G-protein. *FEBS Lett.* **211**:5–9 (1987).
24. Okajima, F., and Y. Kondo. Synergism in cytosolic Ca^{2+} mobilization between bradykinin and agonists for pertussis toxin-sensitive G-protein coupled receptors in NG108–15 cells. *FEBS Lett.* **301**:223–226 (1992).
25. Tomura, H., F. Okajima, and Y. Kondo. Enkephalin induces Ca^{2+} mobilization in single cells of bradykinin-sensitized differentiated neuroblastoma hybridoma (NG108–15) cells. *Neurosci. Lett.* **148**:93–96 (1992).
26. Okajima, F., H. Tomura, and Y. Kondo. Enkephalin activates the phospholipase C/Ca^{2+} system through cross-talk between opioid receptors and P_2-purinergic or bradykinin receptors in NG108–15 cells. *Biochem. J.* **290**:241–247 (1993).
27. Lambert, D. G., A. S. Ghataorre, and S. R. Nahorski. Muscarinic receptor binding characteristics of a human neuroblastoma SK-N-SH and its clones SH-SY5Y and SH-EP1. *Eur. J. Pharmacol.* **165**:71–77 (1989).
28. Whitham, E. M., R. A. J. Challiss, and S. R. Nahorski. M_3 muscarinic cholinoceptors are linked to phosphoinositide metabolism in rat cerebellar granule cells. *Eur. J. Pharmacol.* **206**:181–189 (1991).
29. Abraham, E. H., A. G. Prat, L. Gerweck, T. Seneveratne, R. J. Arceci, R. Kramer, G. Guidotti, and H. F. Cantiello. The multidrug resistance (*mdr1*) gene product functions as an ATP channel. *Proc. Natl. Acad. Sci. USA* **90**:312–316 (1993).
30. Rhee, S. G., and K. D. Choi. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J. Biol. Chem.* **267**:12393–12396 (1992).
31. Sternweis, P. C., and A. V. Smrcka. Regulation of phospholipase C by G proteins. *Trends. Biochem. Sci.* **17**:502–506 (1992).
32. Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton. Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature (Lond.)* **350**:516–518 (1991).
33. Camps, M., A. Carozzi, P. Schnabel, A. Scheer, P. J. Parker, and P. Gierschik. Isozyme-selective stimulation of phospholipase C-β2 by G protein $\beta\gamma$-subunits. *Nature (Lond.)* **360**:684–686 (1992).
34. Katz, A., D. Wu, and M. I. Simon. Subunits $\beta\gamma$ of heterotrimeric G protein activate β2 isoform of phospholipase C. *Nature (Lond.)* **360**:686–689 (1992).
35. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1989).
36. Boyer, J. L., G. L. Waldo, T. Evans, J. K. Northup, C. P. Downes, and T. K. Harden. Modification of AlF_4^-- and receptor-stimulated phospholipase C activity by G-protein $\beta\gamma$ subunits. *J. Biol. Chem.* **264**:13917–13922 (1989).
37. Linden, J. Structure and function of A_1 adenosine receptors. *FASEB J.* **5**:2668–2676 (1991).
38. Channon, J. Y., and C. C. Leslie. A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.* **265**:5409–5413 (1990).
39. Diez, E., and S. Mong. Purification of a phospholipase A_2 from human monocytic leukemic U937 cells. *J. Biol. Chem.* **265**:14654–14661 (1990).
40. Clark, J. D., L. L. Lin, R. W. Kriz, C. S. Ramesha, L. A. Sultzman, A. Y. Lin, N. Milona, and J. Knopf. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **65**:1043–1051 (1991).
41. Jelsema, C. L., and J. Axelrod. Stimulation of phospholipase A_2 activity in bovine rod outer segments by the $\beta\gamma$ subunits of transducin and its inhibition by the α subunit. *Proc. Natl. Acad. Sci. USA* **84**:3623–3627 (1987).

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