A transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding protein G_i that amplifies ATP-mediated arachidonic acid release

(phospholipase A_2/phorbol ester/pertussis toxin/cAMP/protein kinase C)

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ABSTRACT ATP is copackaged and coreleased with adrenergic, serotonergic, and cholinergic neurotransmitters, suggesting a possible interaction between the signaling pathways for ATP and these co-released neurotransmitters. Muscarinic m2 and m4, α2-adrenergic, and D2-dopaminergic neurotransmitter receptors, which have in common their ability to inhibit adenylyl cyclase through the inhibitory guanine nucleotide binding protein G_i, were transfected and expressed in Chinese hamster ovary (CHO) cells that contain endogenous ATP receptors coupled to the release of arachidonic acid. Normal functional coupling of m2, m4, α2, and D2 receptors was demonstrated by their ability to inhibit forskolin-stimulated cAMP accumulation with dose–response activities consistent with previous reports for these G_i-coupled receptors. Stimulation of m2, m4, α2, and D2 receptors resulted in an augmentation of ATP-stimulated arachidonic acid release. With the exception of the m4 receptor, none of the receptors tested was able to stimulate arachidonic acid release in the absence of ATP. Potentiation of ATP-stimulated arachidonic acid release was independent of changes in cAMP. The augmentation of ATP-stimulated arachidonic acid release and the inhibition of cAMP accumulation were both blocked by pertussis toxin, an inhibitor of G_i, but with different dose–response characteristics. Inhibition of protein kinase C with staurosporine or long-term pretreatment of the cells with the phorbol ester phorbol 12-myristate 13-acetate blocked the augmentation response. This demonstrates that G_i-coupled inhibitory receptors can amplify ATP-receptor-stimulated arachidonic acid release through a pertussis-toxin-sensitive G protein, independent of their ability to inhibit adenylyl cyclase activity.

After neural stimulation, neurotransmitters are released from storage vesicles and bind to cell surface receptors to initiate transmembrane signaling. ATP has been shown to be packaged with cholinergic, serotonergic, and adrenergic neurotransmitters in storage vesicles and coreleased during the process of nerve stimulation (1). ATP, in addition to its role in intermediary metabolism and cellular energy, can itself act as a neurotransmitter by binding with high affinity to cell surface purinergic receptors on both excitable and nonexcitable cells. The corelease of ATP with neurotransmitters suggests a possible interaction between their signaling pathways.

The cascade of guanine nucleotide binding protein (G protein)-coupled receptor signaling involves the sequential stimulation of receptors, G proteins, and effector enzymes to generate intracellular second messengers, such as cAMP, arachidonic acid, and inositol phosphates. ATP, acting through P_2-purinergic receptors, stimulates phospholipase A_2 and phospholipase C, which results in the liberation of arachidonic acid and inositol phosphates, respectively (25). Arachidonic acid and many of its eicosanoid metabolites have been shown to be important second messengers in both neural and nonneural cells (2, 3) and may play a role in long-term potentiation and synaptic plasticity (4). We have observed (5) that calcium ionophore-stimulated arachidonic acid release can be amplified by transfected inhibitory D_2 receptors in Chinese hamster ovary (CHO) cells. This suggested that an interaction might exist between receptor-mediated arachidonic acid release and inhibitory G_i-coupled receptors (where G_i is the inhibitory G protein). To examine this possibility, CHO cells were selected that contain endogenous ATP receptors coupled to arachidonic acid release (6). CHO cells are devoid of most neurotransmitter receptors yet contain the appropriate G proteins and effector enzymes to allow arachidonic acid, inositol phosphate, and cAMP second messenger production. Neurotransmitter receptors have been expressed in these cells through DNA transfection techniques permitting the study of mechanisms of signal transduction associated with individual receptor subtypes (7, 8). In this study, CHO cells were transfected with genes for muscarinic m2 and m4, α2-adrenergic, and D2-dopaminergic receptors that have been characterized (9–12) and that have in common their coupling to the inhibition of adenylyl cyclase through the G_i protein. All four of these inhibitory G_i-coupled receptors were shown to augment ATP-stimulated arachidonic acid release.

MATERIALS AND METHODS

Materials. Pertussis toxin, staurosporine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem. [5,6,8,9,11,12,14,15-3H(N)]Arachidonic acid was purchased from New England Nuclear. Clonidine and quinpirole were purchased from Research Biochemicals (Natick, MA). (R_P)-Adenosine cyclic 3',5'-phosphorothioate [(R_P)-cAMP[S]] was purchased from Biolog Life Sciences Institute (La Jolla, CA). Reagents used in the radioimmunoassay of cAMP were purchased from Gary Broker (Department of Biochemistry, Georgetown University School of Medicine, Washington DC). All other reagents were purchased from Sigma.

Cell Culture and Stable Expression of Receptor Clones in CHO Cells. CHO cells were obtained from the American Type Culture Collection and maintained as described (8).

Abbreviations: PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; G protein, guanine nucleotide binding protein; G_i protein, inhibitory G protein; (R_P)-cAMP[S], (R_P)-adenosine cyclic 3',5'-phosphorothioate; 3H(N), tritiated.  

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CHO cells that expressed physiologic levels of the m2 receptor (84 fmol/mg of protein) and the m4 receptor (202 fmol/mg of protein) (9) were generously provided by M. R. Brann (National Institute of Neurological Disorders and Stroke) and N. J. Buckley, and T. I. Bonner (National Institute of Mental Health). CHO cells that expressed physiologic levels of the D2 receptor (1–2 pmol/mg of protein) (10, 12) were generously provided by D. R. Sibley (National Institute of Neurological Disorders and Stroke) and L. C. Mahan (National Institute of Mental Health). CHO cells that expressed slightly lower levels of the a2 receptor (64 fmol/mg of protein) were as described (11). No increases in arachidonic acid release, cAMP generation, inositol phosphate release, or intracellular calcium concentrations were observed when untransfected CHO cells or CHO cells transfected with plasmid DNA, without receptor cDNA, were stimulated with muscarinic, α-adrenergic, or dopaminergic agonists (data not shown). In addition, CHO cells do not express endogenous muscarinic, adrenergic, or dopaminergic receptors as determined by radioligand binding (9–12).

**Assay of Arachidonic Acid Release and cAMP Accumulation.** Cells were labeled overnight with [3H]arachidonic acid (0.20 μCi/ml; 1 Ci = 37 GBq) in growth medium and released free arachidonic acid was analyzed as described (7). Radioimmunoassay of cAMP was performed as described (7).

**RESULTS**

**CHO Cells Expressing the m2- and m4-Muscarinic, D2-Dopaminergic, and a2-Adrenergic Receptors Augmented ATP-Stimulated Release of Arachidonic Acid.** We have demonstrated (5) potentiation of ionophore-stimulated arachidonic acid release in CHO cells transfected with an inhibitory dopamine D2 receptor. These studies were undertaken to determine if an interaction exists between ATP-receptor-stimulated arachidonic acid release and transfected inhibitory receptors. We first examined the effect of transfected m2-muscarinic acetylcholine receptors on the generation of arachidonic acid release after stimulation by ATP. ATP-stimulated arachidonic acid release in the CHO cell, but the stimulation of expressed m2 receptors with the muscarinic agonist carbachol, in the absence of ATP, failed to generate arachidonic acid. Stimulation of the m2 receptor after the addition of ATP markedly potentiated ATP-stimulated release of arachidonic acid (Fig. 1). Addition of the muscarinic antagonist atropine blocked the carbachol-mediated augmentation of arachidonic acid release but had no effect on ATP-stimulated arachidonic acid release, suggesting a muscarinic-receptor-mediated process (data not shown). As expected, the m2 receptor inhibited forskolin-stimulated adenylate cyclase activity (Fig. 1). Other receptors associated with the inhibition of adenylate cyclase were tested for their ability to augment ATP-stimulated release of arachidonic acid. CHO cells expressing m4-muscarinic, D2-dopaminergic, and a2-adrenergic receptors also augmented ATP-stimulated release of arachidonic acid (Fig. 2), suggesting commonality of this response with receptors that inhibit adenylate cyclase activity. Maximal stimulation of D2 and a2 receptors had no effect on arachidonic acid release in the absence of ATP (data not shown), and the m4 receptor caused a modest increase in arachidonic acid release (38% above basal). Therefore, receptors that normally inhibit adenylate cyclase can amplify purinergic-receptor-stimulated arachidonic acid release.

**Potentiation of ATP-Stimulated Arachidonic Acid Release Is Independent of Changes in cAMP.** Since the known second messenger pathway common among the transfected G (i) receptors tested is the inhibition of adenylate cyclase, it is possible that decreases in cAMP levels may be required for amplification of ATP-stimulated arachidonic acid release. It would follow that increases in cAMP should reverse the augmentation effect. Stable analogs of cAMP or agents that stimulate cAMP production were added to CHO cells expressing the m2 receptor to overcome the augmentation of arachidonic acid release (Table 1). Addition of the nonhydrolyzable cAMP analogs 8-(4-chlorophenylthio)-cAMP and 8-bromo-cAMP had no effect on the inhibitory-receptor-mediated amplification of arachidonic acid. Similar results were seen for forskolin, which directly activates adenylate cyclase, and for prostaglandin E2, which stimulates cAMP generation through an endogenous receptor coupled to the

**Fig. 1.** Stimulation of m2-muscarinic receptor augments ATP-stimulated arachidonic acid release. Clonal CHO cells, transfected with and expressing the m2-muscarinic receptor, were prelabeled overnight with [3H]arachidonic acid and arachidonic acid release was measured over 15 min. Maximal ATP (5 μM)-stimulated arachidonic acid release is shown (EC50, 958 ± 4 nM). Carbachol, a muscarinic agonist, augmented ATP-stimulated arachidonic acid release with an estimated EC50 of 871 ± 24 nM. Data are the mean ± SEM of three experiments performed in triplicate. The EC50 value for m2-mediated inhibition of forskolin-stimulated cAMP generation using carbachol was 537 ± 24 nM. EC50 values were estimated by nonlinear regression of primary data. BSL, basal level.

**Fig. 2.** Stimulation of D2-dopaminergic, m4-muscarinic, and a2-adrenergic receptors augments ATP-stimulated arachidonic acid release. Clonal CHO cell lines individually transfected with and expressing the D2, m4, and a2 receptors were stimulated with the selective agonists quinpirole, carbachol, and clonidine, respectively. Arachidonic acid release was measured over 15 min. Data represents the percent augmentation of arachidonic acid release over maximal ATP stimulation (5 μM). Estimated EC50 values for percent augmentation are as follows: D2, 11 ± 0.1 nM; m4, 338 ± 3 nM; a2, 129 ± 0.1 nM. Data are the mean ± SEM of three experiments performed in triplicate. EC50 values for the inhibition of forskolin-stimulated cAMP generation using the selective agonists quinpirole, carbachol, and clonidine, respectively, were as follows: D2, 2.0 ± 0.01 nM; m4, 34 ± 1.1 nM; a2, 2.6 ± 0.1 nM.
Table 1. Changes in cAMP levels do not effect ATP- or m2-receptor-mediated augmentation of arachidonic acid release

<table>
<thead>
<tr>
<th>Addition</th>
<th>Arachidonic acid release, cpm</th>
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<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>None (control)</td>
<td>1639 ± 8</td>
</tr>
<tr>
<td>Prostaglandin E2 (1 μM)</td>
<td>1682 ± 4</td>
</tr>
<tr>
<td>Forskolin (500 nM)</td>
<td>1565 ± 28</td>
</tr>
<tr>
<td>CPT-cAMP (10 μM)</td>
<td>1697 ± 36</td>
</tr>
<tr>
<td>8-Bromo-CAMP (10 μM)</td>
<td>1590 ± 43</td>
</tr>
<tr>
<td>(Rp)-cAMP[S] (1 μM)</td>
<td>1568 ± 18</td>
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Maximal ATP (5 μM)-stimulated arachidonic acid release or carbachol (10 μM)-mediated augmentation of maximal ATP-stimulated arachidonic acid release was assayed after a 10-min preincubation with various agents as indicated. Prostaglandin E2 (1 μM) stimulated cAMP generation (basal = 2.1 ± 0.1 and prostaglandin E2 = 12.3 ± 0.9 pmol/ml) through endogenous receptors. Forskolin (500 nM) stimulated cAMP generation (basal = 1.6 ± 0.1 and forskolin = 12.6 ± 0.9 pmol/ml) through direct activation of adenylyl cyclase. Data are the mean ± SEM of three experiments performed in triplicate. CPT-cAMP, 8-(4-chlorophenylthio)-cAMP.

stimulatory G protein G1. Decreases in cAMP levels within the cell may result in a decrease in cAMP-dependent protein kinase A activity, which may play a role in the amplification response. (Rp)-cAMP[S], a membrane-permeable inhibitor of cAMP-dependent protein kinase A (3), was added to mimic the augmentation of ATP-stimulated arachidonic acid release. (Rp)-cAMP[S] had no effect on ATP-stimulated arachidonic acid release or the augmentation. Thus changes in cAMP had no influence on m2-muscarinic-receptor-mediated amplification of ATP-stimulated arachidonic acid release. Similar results were seen for D2 and a2 receptors (data not shown). The E50 values for inhibition of forskolin-stimulated cyclase were lower than E50 values for augmentation of ATP-stimulated arachidonic acid release for all inhibitory receptors tested (see Figs. 1 and 2), further dissociating the augmentation response from changes in cAMP.

Potentiation of ATP-Stimulated Arachidonic Acid Release Is Sensitive to Pertussis Toxin. The transfected receptors used in this study inhibit adenylyl cyclase activity through the G protein G1. Pertussis toxin treatment blocks the action of Gi by inducing ADP-ribosylation and persistent association of the G subunit heterotrimer and, therefore, preventing the G subunit from transducing the inhibitory signal to adenylyl cyclase (14). Pertussis toxin has also been shown to inhibit receptor-stimulated arachidonic acid release, suggesting the involvement of G-like proteins in this signaling pathway (6, 15). After 12 hr of preincubation, pertussis toxin completely inhibited m2-receptor-mediated potentiation of arachidonic acid release (Fig. 3) but had no effect on basal or ATP-stimulated arachidonic acid release (data not shown). This suggests the involvement of a pertussis-sensitive-G-like protein in the amplification response. Pertussis toxin also reversed carbachol-mediated inhibition of forskolin-stimulated adenylyl cyclase consistent with a previous report (16). The m2-receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase was more sensitive to inhibition by pertussis toxin than was augmentation of arachidonic acid release, as shown by the left shift of the dose-response curve in Fig. 3. Although both responses are sensitive to pertussis toxin, the differential sensitivities suggest either that distinct G-like proteins are involved or that the augmentation response is a more complex multistep process.

Inhibitors of Protein Kinase C Block the Potentiation of ATP-Stimulated Arachidonic Acid Release. Receptor-mediated stimulation of phospholipase A2 activity in CHO cells has been shown to involve the activation of protein kinase C (8, 17). Staurosporine, a selective inhibitor of protein kinase C (18), blocked m2-, D2-, and a2-mediated augmentation of arachidonic acid release (Fig. 4) but had no significant effect on basal or ATP-stimulated arachidonic acid release (data not shown). Further evidence of protein kinase C involvement in the muscarinic-m2-receptor-mediated augmentation response was shown by preincubating the CHO cells with the phorbol ester PMA to desensitize protein kinase C (7) (Fig. 5). After 4 hr of preincubation, PMA inhibited the m2-receptor-mediated augmentation of arachidonic acid release. Under the same conditions, the inactive phorbol ester 4a-phorbol had no effect on the augmentation response (data not shown). These data suggest a role for protein kinase C in the augmentation of arachidonic acid release mediated by inhibitory G1-coupled receptors.

**DISCUSSION**

Our results demonstrate that inhibitory G1-coupled receptors can amplify ATP-receptor-mediated release of arachidonic acid through a pertussis-sensitive process.

![Fig. 3. Pertussis toxin inhibits m2 receptor augmentation of ATP-stimulated arachidonic acid release and reverses m2-receptor inhibition of cAMP generation. Clonal CHO cells expressing the m2-muscarinic receptor were treated for 12 hr with increasing concentrations of pertussis toxin. CHO cells expressing the m2 receptor were then assayed for either carbachol (100 μM)-stimulated inhibition of forskolin (500 nM)-stimulated cAMP accumulation or carbachol (10 μM)-mediated augmentation of maximal ATP (5 μM)-stimulated release of arachidonic acid. Data are the mean ± SEM of three experiments performed in triplicate.](image-url)

![Fig. 4. Staurosporine, an inhibitor of protein kinase C, blocked m2, D2, and a2 receptor augmentation of ATP-stimulated arachidonic acid release. Clonal CHO cells expressing the m2, D2, or a2 receptors were stimulated with ATP (5 μM) alone or with ATP in combination with 10 μM carbachol (CC), 100 nM dopamine (DA), or 10 μM norepinephrine (NE), and arachidonic acid release was measured after 15 min. Data represented by the solid bars were preincubated with staurosporine (1 μM for 10 min); data represented by open bars were not. Data are the mean ± SEM of three experiments performed in triplicate. Staurosporine inhibited the augmentation response with an IC50 of 50 nM and the complete inhibition at 1 μM is shown.](image-url)
Figure 5. Pretreatment (4 hr) with the phorbol ester PMA blocks m2 receptor augmentation of ATP-stimulated arachidonic acid release. Clonal CHO cells expressing m2 receptors were stimulated with ATP (5 μM) alone or with ATP in combination with either 10 μM carbachol (CC) or carbachol plus 100 nM PMA and arachidonic acid release was measured after 15 min. CHO cells were preincubated with vehicle or PMA for 4 hr to desensitize protein kinase C. Data are the mean ± SEM of three experiments performed in triplicate.

Acid. Except for a modest increase seen for the m4 receptor, the receptors tested were unable to stimulate arachidonic acid release in the absence of ATP. The potentiation of arachidonic acid was independent of changes in cAMP, suggesting that an alternate signaling pathway might be operating to induce this response. The amplification of arachidonic acid release was dependent on a pertussis-toxin-sensitive G protein that was less sensitive to the toxin than was the inhibition of cAMP. These results further suggest that an alternate G-protein might be involved in the potentiation response. Similar conclusions were suggested in a previous study using CHO cells, in which low concentrations of pertussis toxin differentially inhibited muscarinic-m2-receptor-mediated inositol phosphate release and cAMP inhibition, whereas at higher concentrations, complete ADP-ribosylation of pertussis-sensitive substrates was observed (19).

Inhibition of the potentiation response by two inhibitors of protein kinase C, staurosporine and long-term preincubation with PMA, suggests that protein kinase C may be activated by inhibitory G-protein-coupled receptors, although evidence of direct activation by these receptors has yet to be demonstrated. Overexpression of G-protein-coupled receptors that inhibit adenylate cyclase in fibroblasts (19), HeLa cells (20), or Ltk- cells (21) resulted in stimulation of phospholipase C that would presumably liberate diacylglycerol and stimulate protein kinase C. This mechanism of protein kinase C activation is not likely in our studies since no significant increase in inositol phosphate release was seen for the expression levels of receptors tested. Direct release of diacylglycerol by phosphatidylcholine-specific phospholipase C, independent of inositol phosphate release, cannot be ruled out.

It is becoming apparent that activation of multiple receptors is involved in inducing (22), enhancing (23), and inhibiting (24) receptor-mediated signaling processes. Our results demonstrate that the inhibitory m2- and m4-muscarinic, α2-adrenergic, and D2-dopaminergic receptors augment ATP-stimulated arachidonic acid release. This association between signaling pathways for neurotransmitters that are copackaged and coreleased with ATP would result in the enhanced release of arachidonic acid and its many bioactive eicosanoid metabolites. Modulation of ATP-stimulated arachidonic acid release may be a common feature of G-protein-coupled inhibitory receptors.

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