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Sensitization of Adenylyl Cyclase by P_2 Purinergic and M5 Muscarinic Receptor Agonists in L Cells

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SUMMARY

Many hormones have been shown to activate phospholipase C, which results in the hydrolysis of membrane polyphosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP_2). Two second messengers are known to be produced by PIP_2 hydrolysis, 1,2-diacylglycerol, an endogenous activator of a family of enzymes called protein kinase C (PKCs), and inositol 1,4,5-trisphosphate, which raises free levels of intracellular Ca^{2+}. Treatment of various cells with 4β-phorbol 12-myristate 13-acetate (PMA), a specific exogenous activator of PKCs, causes an enhancement or sensitization of adenylyl cyclase activities. This finding prompted us to examine the effects of direct hormonal activation of PIP_2 hydrolysis on the sensitization of adenylyl cyclase. Liao *et al.* [*J. Biol. Chem.* **265**:11273–11284 (1990)] have shown that P_2 purinergic receptor agonists such as ATP and muscarinic receptor agonists such as carbachol stimulate PIP_2 hydrolysis in L cells expressing the M5 muscarinic acetylcholine receptor. We investigated the effects of these hormones on adenylyl cyclase and contrasted these effects with the sensitizing effects of PMA. We found that ATP pretreatment of two different types of L cells resulted in a rapid 50–150% sensitization of prostaglandin E_1-, epinephrine-, and forskolin-stimulated adenylyl cyclase activity, with an EC_{50} of 3 μM ATP. This effect was qualitatively similar to that caused by 10 nM PMA. The enhancement of adenylyl cyclase activity was associated with an increase in the V_{max} for hormonal stimulation and with a lack of significant effects of ATP on the EC_{50}. The effect was completely eliminated when adenylyl cyclase was assayed in the presence of high free Mg^{2+} levels (10 mM). Down-regulation of PKCs with long term PMA treatment did not affect the ATP-induced sensitization of adenylyl cyclase, although the PMA-induced sensitization of adenylyl cyclase was eliminated. In contrast to the effects of ATP and PMA, treatment of the cells with carbachol alone had no effect on adenylyl cyclase; however, in combination with nanomolar concentrations of PMA, synergism of the sensitization of adenylyl cyclase was observed. These data indicate that the activation of P_2 purinergic receptors by ATP, and possibly activation of M5 muscarinic receptors by carbachol, may be important in the signal transduction pathways leading to the increases in the responsiveness of hormone-stimulated adenylyl cyclase.

Many reports have indicated that the adenylyl cyclase system may have interactive roles with the components of other second messenger systems, which contribute to its overall regulation. Studies with phorbol esters, compounds that are believed to be the exogenous specific activators of a family of enzymes called PKCs, have suggested that PKCs may phosphorylate components of adenylyl cyclase to alter its function (1–12). For example, we recently reported that, in mouse L cells transfected with the gene for the hamster βAR, the phorbol ester PMA caused both desensitization and sensitization of epinephrine-stimulated adenylyl cyclase activity (12). The desensitization appeared to be caused by a PKC-induced phosphorylation of the βAR, because the hormonally stimulated adenylyl cyclase activity failed to desensitize in the cells expressing a mutant βAR lacking the PKC consensus site for phosphorylation on the third intracellular loop (12). The sensitization of adenylyl cyclase was most consistent with a modification of G_i or the catalyst of adenylyl cyclase, because epinephrine, PGE_1, and forskolin stimulations of adenylyl cyclase were equally sensitized and G_i-mediated inhibition was reduced 50–60% (12). The PMA-induced sensitization of adenylyl cyclase suggested that the hormones that activate PLC or PLD, resulting in the production of the endogenous activators of PKCs, may also sensitize adenylyl cyclase.

Although PMA has been widely used to evaluate the role of PKCs in biochemical processes, its effects on second messenger

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ABBREVIATIONS: PKC, protein kinase C; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; PMA, 4β-phorbol 12-myristate 13-acetate; βAR, β-adrenergic receptor; PGE_1, prostaglandin E_1; PLD, phospholipase D; M5mAChR, M5 subtype of the muscarinic acetylcholine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; App (NH)p, adenosine 5'-(β,γ-imido)triphosphate; ADP-β-S, adenosine 5'-*O*-(β-thio)diphosphate.

function are often not reproduced when cells are treated with hormones that stimulate the synthesis of DAGs (13–16), the endogenous activators of PKCs. For example, many have reported PMA-induced sensitization and desensitization of cAMP accumulation (2, 3, 6, 7, 17–19) and adenylyl cyclase activities (1, 4–7, 9–12). However, although there have also been reports of the sensitization of cAMP accumulation in various types of intact cells by the hormones that activate PLC, such as histamine, carbachol, vasopressin, and angiotensin (13, 15, 18, 20–22), convincing data demonstrating a direct effect of these hormones on adenylyl cyclase function are lacking. It is possible that different mechanisms cause the sensitization of cAMP accumulation reported by others (13, 15, 18, 20–22) and the sensitization of adenylyl cyclase activity in membranes that we report here. A more complete discussion of the various hormonal stimuli that cause an enhancement of cAMP levels is presented by Clark (8) and by Johnson and Toews (15).

Having previously characterized the effects of PMA on adenylyl cyclase in L cells (12), we became interested in comparing the effects of hormones that activate PLC with those caused by PMA. Liao *et al.* (23) recently reported that treatment of L cells with ATP results in the production of inositol trisphosphate and the release of intracellular Ca^{2+} and arachidonate. These effects of ATP are believed to occur through activation of the P_2 subtype of purinergic receptors, which are present in the parental (nontransfected) cells. This group also stably transfected L cells with the gene for the m5mAChR (LM5 cells) and demonstrated that carbachol also stimulates intracellular Ca^{2+} release and arachidonate production. Furthermore, carbachol is approximately 5–10-fold more effective than ATP at stimulating the production of polyphosphoinositides (23, 24). This characterization of the effects of ATP and carbachol on the PIP_2-specific PLC/Ca^{2+} pathway led us to determine the effects of its activation on adenylyl cyclase function in L cells.

We report here that treatment of L cells with ATP caused a sensitization of hormone- and forskolin-stimulated adenylyl cyclase activities. The augmentation of adenylyl cyclase caused by ATP was similar to that caused by PMA in several important characteristics (Mg^{2+} sensitivity, loss of G_i-mediated inhibition, and stability) but differed in that ATP did not cause a desensitization (increased K_{act}) of hormonal stimulation of adenylyl cyclase. Further, we found that down-regulation of PKCs by overnight treatment with PMA completely eliminated the sensitization caused by PMA but had no effect on that induced by ATP. In contrast, treatment of LM5 cells with carbachol alone had no effect on adenylyl cyclase function. This was surprising, because carbachol was shown to be a much more effective stimulator of phosphatidylinositol-PLC activity than ATP (23). Although carbachol had no effect alone on adenylyl cyclase, it did act synergistically with relatively low concentrations of PMA to sensitize adenylyl cyclase.

Materials and Methods

Description of cell lines. Two L cell lines were used in our studies of the ATP-induced sensitization. The first cell line (LβAR), obtained from Dr. Richard Dixon (Texas Cardiac Institute, Houston, TX) and Dr. Catherine Strader (Merck, Sharpe, and Dohme, Rahway, NJ), had been transfected with the gene for the wild-type hamster βAR as previously described (25). The vector carrying the βAR gene also contained a gene that conveyed geneticin resistance. LβAR cells were cultured in Corning T150 tissue culture flasks (Figs. 2, 3, 4, 6, and 7 and Table 1), as previously described, in the presence of geneticin (12) or in suspension (Figs. 5 and 9) without geneticin. In general, LβAR cells grown in suspension yielded a slightly greater ATP-induced sensitization of adenylyl cyclase than LβAR cells grown in monolayers.

The second L cell line used in our studies, LM5 (Figs. 1 and 8), had been stably transfected with the gene for the rat M5mAChR by Liao *et al.* (24). Briefly, L cell mutants deficient in the thymidine kinase gene were co-transfected with the cloned M5mAChR gene and the herpes simplex virus thymidine kinase gene (24). The LM5 cells were grown in monolayers in minimum essential medium α with hypoxanthine/aminopterin/thymidine selection supplement (GIBCO), 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum.

The parental L cells do not express βARs or M5mAChRs. They do express both P_2 purinergic and PGE_1 receptors. When the effects of carbachol on adenylyl cyclase function were examined, LM5 cells were used, and only PGE_1-stimulated adenylyl could be studied. In LβAR cells the effects of ATP on both epinephrine- and PGE_1-stimulated adenylyl cyclase activities were monitored, but carbachol-induced effects could not be studied.

The expression of the M5mAChR in LM5 cells was confirmed by *N*-[^{3}H]methylscopolamine binding and carbachol stimulation of inositol phosphate production, as previously described (23).

Cell incubations and membrane preparations. Cells in complete growth medium in 150-mm^2 flasks were treated at 37° with ATP, carbachol, H_2O, PMA, or ≤0.2% dimethyl sulfoxide, as indicated. After

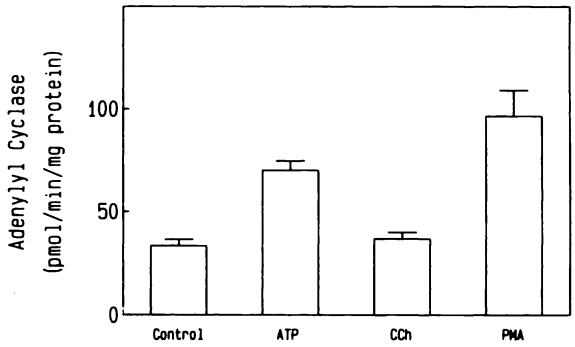

Fig. 1. Sensitization of PGE_1-stimulated adenylyl cyclase in LM5 cells. Preconfluent LM5 cells were treated for 12 min at 37° with 30 μM carbachol (*CCh*), 30 μM ATP, or 10 nM PMA, as indicated. Membranes were prepared, and 5 μM PGE_1-stimulated adenylyl cyclase activity was assayed. Data are plotted as the mean ± standard error of adenylyl cyclase activities from multiple experiments, each performed in triplicate. The number of experiments conducted for control, ATP, and carbachol groups was 14 and for PMA was 4.

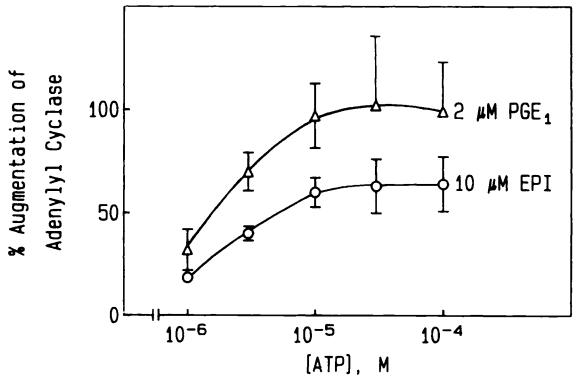

Fig. 2. Concentration-response of ATP-induced sensitization of adenylyl cyclase in LβAR cells. Preconfluent LβAR cells grown in T-150 flasks were treated with the indicated concentrations of ATP for 10 min at 37° in growth medium. Membranes were prepared, and 10 μM epinephrine- and 2 μM PGE_1-stimulated adenylyl cyclase activities were assayed. The data shown represent the mean ± standard error of the percentage of augmentation of treated versus control adenylyl cyclase activities from three experiments, each performed in triplicate. *Epi*, epinephrine.

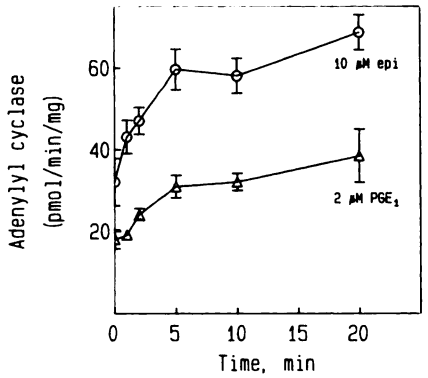

Fig. 3. Time course of ATP-induced sensitization of adenylyl cyclase in LβAR cells. Preconfluent LβAR cells grown in 150-mm² flasks were treated with 30 μM ATP for the indicated times at 37° in growth medium. Membranes were prepared, and 10 μM epinephrine- and 2.0 μM PGE_1-stimulated adenylyl cyclase activities were assayed. The data shown represent the mean ± standard error of adenylyl cyclase activities from four experiments, normalized to the average of the control (0 time).

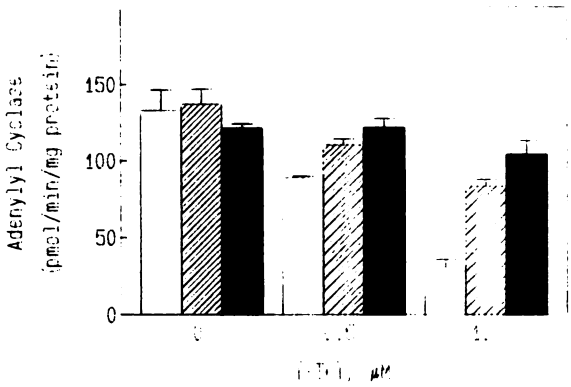

Fig. 5. Effect of GTP on the observation of ATP-induced sensitization of forskolin-stimulated adenylyl cyclase. LβAR cells were grown in suspension and treated for 20 min at 37° with 0.02% dimethyl sulfoxide (□), 100 μM ATP (▨), or 0.5 μM PMA (■). To terminate treatments, cells were chilled for 3 min in an ice bath with constant swirling, concentrated by centrifugation (600 × *g* for 3 min), washed once with buffered saline, and resuspended in HME (see Materials and Methods) plus 10 μg/ml leupeptin. Membranes were prepared and 25 μM forskolin-stimulated adenylyl cyclase was assayed in the presence of the indicated concentrations of GTP. Data are plotted as the mean ± standard error of triplicate determinations from a single experiment.

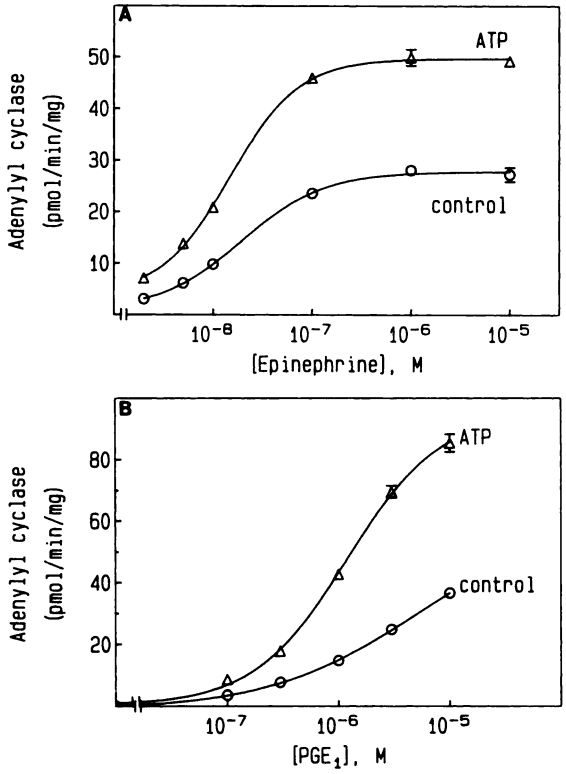

Fig. 4. Effects of ATP treatment on the EC_{50} and V_{max} for hormone-stimulated adenylyl cyclase in LβAR cells. LβAR cells were treated with or without 30 μM ATP for 10 min, membranes were prepared, and adenylyl cyclase stimulated by epinephrine (A) or PGE_1 (B) was assayed. The K_{act} (EC_{50}) and V_{max} values for hormone-stimulated adenylyl cyclase for the experiments shown were determined using Eadie-Hofstee analysis. The K_{act} values for epinephrine-stimulated adenylyl cyclase activity for control and ATP groups were 16.8 and 12.7 nM, respectively. The V_{max} values for control and ATP groups were 27.6 and 50.0 pmol/min/mg, respectively. The K_{act} values for PGE_1-stimulated adenylyl cyclase for control and ATP groups were 0.97 and 1.04 μM, respectively. The V_{max} values for PGE_1-stimulated adenylyl cyclase for control and ATP treatment groups were 34.5 and 85.2 pmol/min/mg, respectively. Data are plotted as the mean ± standard error of triplicate determinations from a single experiment.

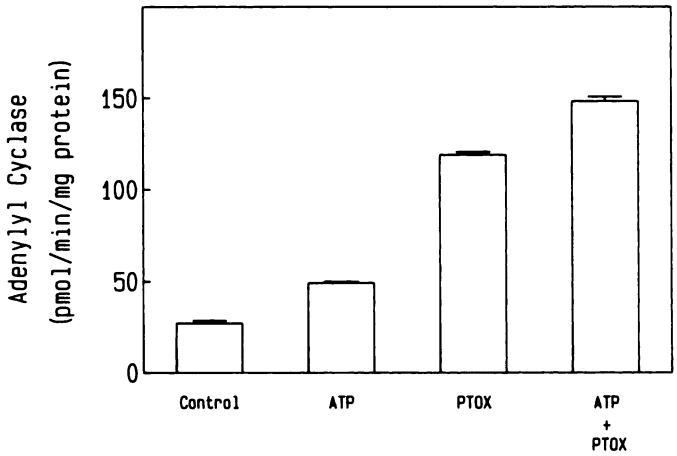

Fig. 6. Effects of pertussis toxin pretreatment on the ATP-induced sensitization of adenylyl cyclase. LβAR cells were cultured overnight (22 hr) in the presence or absence of 20 ng/ml pertussis toxin (*PTOX*). Cells were then treated for 10 min with or without 30 μM ATP. Membranes were isolated and 10 μM epinephrine-stimulated adenylyl cyclase activity was assayed. Data are plotted as the mean ± standard error of triplicate determinations from a single experiment.

the pretreatments, the cells were washed twice with cold HME buffer (20 mM HEPES, pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 2 mM tetrasodium pyrophosphate, 10 μg/ml trypsin inhibitor, 0.1 mg/ml bovine serum albumin), scraped in HME plus 10 μg/ml leupeptin, and homogenized in a type B Dounce homogenizer. Membranes were prepared over sucrose gradients in 20 mM HEPES, pH 8.0, 1 mM EDTA, as previously described (26). It was not necessary to include benzamidine, tetrasodium pyrophosphate, or trypsin inhibitor to observe the ATP-induced sensitization of adenylyl cyclase.

Adenylyl cyclase assays. Adenylyl cyclase was assayed as previously described (26). The free Mg^{2+} concentration, unless otherwise indicated, was 0.3 mM, as calculated by the method of Iyengar and Birnbaumer (27). Other assay components were 40 mM HEPES, pH 7.7, 1 mM EDTA, 8 mM creatinine phosphate, 16 units/ml creatine phosphokinase, 0.05 mM ATP, 10 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, 2 μCi of [α-^{32}P]ATP, and 15–30 μg of membrane protein, in a final volume of 100 μl. All adenylyl cyclase activities are expressed

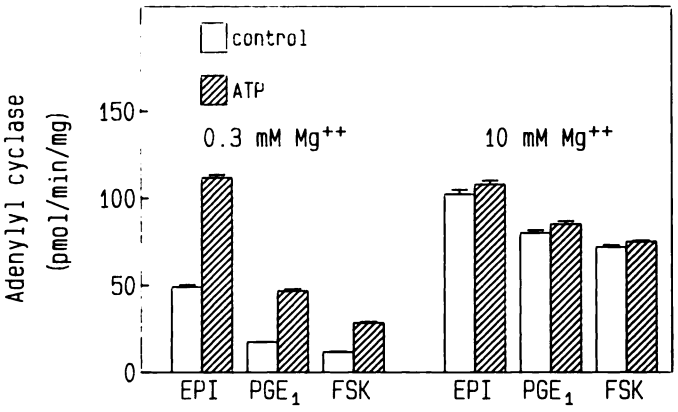

Fig. 7. Effects of Mg^{2+} on the ATP-induced sensitization of adenylyl cyclase. LβAR cells were treated for 20 min at 37° with H_2O (□) or 100 μM ATP (▨). Membranes were prepared and epinephrine-, PGE_1-, and forskolin-stimulated adenylyl cyclase activities were assayed in the presence of either 0.3 mM or 10 mM free Mg^{2+}. The data are the mean ± standard error of triplicate determinations from a single experiment. *EPI*, epinephrine; *FSK*, forskolin.

TABLE 1

Effects of various purinergic receptor agonists on adenylyl cyclase

LβAR cells were treated for 10 min at 37° with the indicated purinergic receptor agonist at a concentration of 30 μM. Membranes were isolated and 2 μM PGE_1-stimulated adenylyl cyclase was assayed. The values shown are the means ± standard errors of triplicate determinations from a single experiment. The numbers in parentheses represent the percentage of the control.

Agonist	Adenylyl cyclase activity
	pmol/mg/min
None	13.0 ± 0.1 (100%)
ADP	14.5 ± 0.2 (112%)
ADP-β-S	13.1 ± 0.3 (101%)
App(NH)p	13.0 ± 0.1 (100%)
ATP	25.2 ± 0.2 (194%)
UTP	21.1 ± 0.4 (162%)

as the means ± standard errors of triplicate determinations. In the case of multiple experiments, data are expressed as means ± standard errors of individual experiment means.

Down-regulation of PKC and isolation of cytosol and particulate fractions. LβAR cells were grown in suspension and treated overnight at 37° in growth medium with or without 0.2 μM PMA. Cells (3×10^8/treatment group) were washed twice with phosphate-buffered saline and homogenized on ice in 5 ml of lysis buffer composed of 20 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM EGTA, 2 mM DTT, 1 mM benzamidine, and 10 μg/ml leupeptin, at 0–4°. Homogenates were centrifuged at 35,000 × *g* for 35 min at 4°. The supernatants were used as the cytosolic fractions. The pellets were resuspended in 5 ml of cold lysis buffer using a tuberculin syringe and a 23-gauge needle and were used as particulate fractions.

Purification of PKC from cytosolic and particulate functions. For experiments with small-scale DE52 chromatography, cytosolic and particulate fractions were applied to a 2-ml DE52 column equilibrated with column buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 1 mM benzamidine). The column was washed with 10 ml of column buffer and then eluted with 5 ml of column buffer containing 0.5 M NaCl. Fractions (1-ml) were collected and assayed for PKC-mediated ^{32}P incorporation into histone IIIs: as previously described (28), with modifications. Briefly, the PKC assay was carried out in a total volume of 100 μl and contained 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.8 mM EDTA, 0.8 mM EGTA, 1.5 mM $CaCl_2$, 5 mM DTT, 1 mg/ml histone IIIs, 0.05 mM ATP, [γ-^{32}P]ATP (200–400 cpm/pmol), 1 μM PMA, 0.2 or 3 μg of phosphatidylserine (both concentrations yielded the same response), and 40 μl of enzyme fraction. Incubations were terminated on P81 phosphocellulose filters (Whatman).

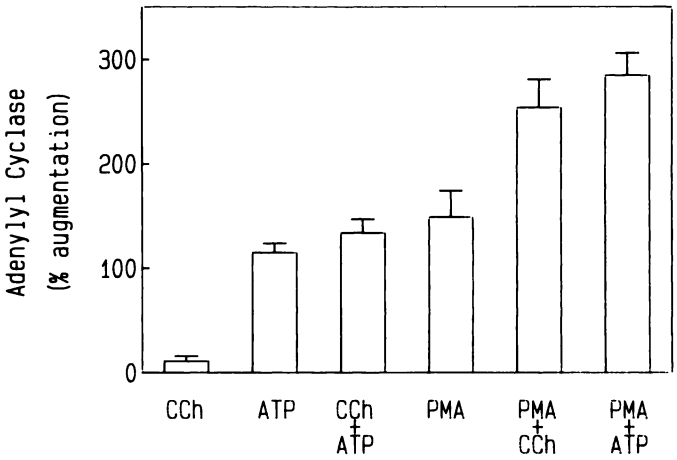

Fig. 8. Effects of various combinations of carbachol, ATP, and PMA on PGE_1-stimulated adenylyl cyclase in LM5 cells. Preconfluent LM5 cells were treated for 12 min at 37° with 30 μM carbachol (*CCh*), 30 μM ATP, carbachol plus ATP, 10 nM PMA, PMA plus carbachol, or PMA plus ATP, as indicated. Membranes were prepared and 5 μM PGE_1-stimulated adenylyl cyclase activity was assayed. Data are plotted as the mean ± standard error of the percentage of augmentation of treated versus control cells from multiple experiments, each assayed in triplicate. The number of experiments performed was as follows: for control, ATP, and carbachol groups, 14; for PMA, ATP plus PMA, and carbachol plus PMA groups, four; and for ATP plus carbachol, five. The average control PGE_1-stimulated adenylyl cyclase activity was 33.6 ± 2.9 pmol/min/mg of protein.

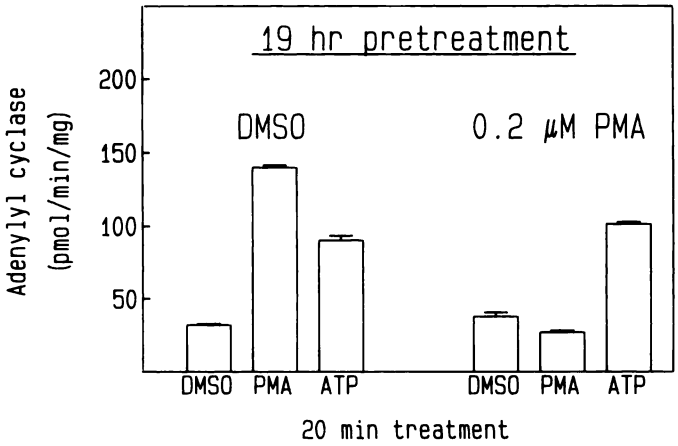

Fig. 9. Long term PMA treatment differentiates PMA- versus ATP-induced sensitization of adenylyl cyclase. LβAR cells grown in suspension at 2×10^5 cells/ml were treated with 0.2 μM PMA in dimethyl sulfoxide or with 0.02% dimethyl sulfoxide (*DMSO*) for 19 hr at 37° and were then treated, without washing, for 20 min at 37° with either H_2O, 0.5 μM PMA, or 100 μM ATP. Treatments were terminated as described in the legend to Fig. 5. Cells were homogenized, membranes were isolated, and 2 μM PGE_1-stimulated adenylyl cyclase was assayed (26). Data are plotted as the mean ± standard error of triplicate determinations from a single experiment, typical of two identical experiments.

Filters were washed twice with H_2O, dried under a heating lamp, and counted in 5 ml of Betafluor (National Diagnostics) scintillation cocktail for 5 min. PKC fractions isolated from cells treated with or without 0.2 μM PMA overnight were assayed, and the percentage of down-regulation of PKC activity was determined.

Large-scale DE52 purification of PKC from cytosolic and particulate fractions used methodologies similar to those used for the small-scale DE52 experiments, with the following exceptions: 1) 1.8×10^9 cells were used per treatment group; 2) cells were homogenized in 25 ml of lysis buffer; 3) a 30-ml DE52 column was used to purify PKC, and after the samples were applied the column was washed with 150 ml of

column buffer; 4) PKC was eluted with a linear gradient of 0–0.5 M NaCl; and 5) 5-ml PKC fractions were collected, and 20 μl of a 1/3 diluted fraction were assayed for PKC activity.

Results

Effects of ATP, carbachol, and PMA on PGE_1-stimulated adenylyl cyclase activity. To determine whether treatment of L cells with hormones coupled to PLC could have effects on adenylyl cyclase and to compare their effects on adenylyl cyclase with those caused by treatment with PMA, LM5 cells were treated with ATP, carbachol, or PMA. As shown in Fig. 1, 30 μM ATP caused an enhancement of PGE_1-stimulated adenylyl cyclase activity that qualitatively resembled the sensitizing effects of 10 nM PMA. The average augmentation of 5 μM PGE_1-stimulated adenylyl cyclase activity caused by pretreatment with 30 μM ATP was 115 ± 9% (14 experiments). In contrast, treatment of LM5 cells with carbachol alone had no significant effect on PGE_1-stimulated adenylyl cyclase (11 ± 5%) (14 experiments). Basal adenylyl cyclase activity (<1 pmol/min/mg) did not appear to be altered by the pretreatments, although at these low levels of activity, measurements are difficult to discern from background.

Characterization of the effects of ATP pretreatment on hormone-stimulated adenylyl cyclase. In order to determine whether the effect of ATP was selective for PGE_1 stimulation of adenylyl cyclase, L cells expressing the βAR were used for further characterization. Fig. 2 shows that the EC_{50} for the ATP enhancement of epinephrine- and PGE_1-stimulated adenylyl cyclase was approximately 2 μM. Fig. 3 shows that the sensitization of epinephrine- and PGE_1-stimulated adenylyl cyclase activity was maximal within 5 min and remained unchanged for at least 20 min. These data establish that the effects of ATP on the responsitivity of two stimulatory receptors were nearly equivalent.

The effects of ATP treatment on the K_{act} and V_{max} for hormonal stimulation of adenylyl cyclase were determined as described in the legends to Fig. 4, A and B. Data were transformed into Eadie-Hofstee plots, and V_{max} and K_{act} were determined. Treatment of the LβAR cells with 30 μM ATP resulted in average increases in the V_{max} for epinephrine- and PGE_1-stimulated adenylyl cyclase of 100 ± 10% (four experiments) and 113 ± 6% (three experiments), respectively. The K_{act} for epinephrine and PGE_1 stimulations was slightly decreased, 21 ± 5% (four experiments) and 13 ± 3% (three experiments), respectively.

To investigate the reversibility of the sensitization caused by ATP, cells were incubated for 12 min with 30 μM ATP, washed twice with 37° minimum essential medium α, and incubated for 12 min in the absence of ATP. In the LM5 cells the sensitization of 5 μM PGE_1-stimulated adenylyl cyclase was reduced from 74.5 ± 3.5% to 31 ± 15% (two experiments) (59% reversal). Similarly, in LβAR cells sensitization of 10 μM epinephrine-stimulated adenylyl cyclase activity was reduced from 103% to 40% (one experiment) after the 12-min washout period (61% reversal).

Effects of GTP on the ATP-induced augmentation. As can be seen in Fig. 5, the maximal augmentation of adenylyl cyclase by forskolin stimulation was dependent on the presence of 10 μM GTP. ATP reduced the GTP-mediated inhibition of forskolin-stimulated adenylyl cyclase. These data indicate that ATP treatment causes an attenuation of G_i-mediated inhibition of adenylyl cyclase activity similar to that caused by PMA treatment (12). Also, the augmentation of forskolin-stimulated adenylyl cyclase caused by PMA treatment of cells has a similar dependency on GTP. However, it should be noted that the magnitude of the ATP effect in the results shown in Fig. 5 was unusually great; ATP was, on average, about half as effective as 0.5 μM PMA. Observation of the maximal augmentation of epinephrine stimulation of adenylyl cyclase was also dependent on the presence of 10 μM GTP (data not shown).

Effects of pertussis toxin on the ATP-induced sensitization of adenylyl cyclase. To investigate the possibility that the ATP-induced sensitization resulted from an inhibition of G_i function, the effects of ATP and pertussis toxin treatment on the hormonal stimulation of adenylyl cyclase in LβAR cells were compared (Fig. 6). ATP caused the expected sensitization of adenylyl cyclase, and pertussis toxin had a much greater effect. The effects of ATP and pertussis toxin were additive. The percentages of augmentation of 10 μM epinephrine-stimulated adenylyl cyclase activity caused by ATP, pertussis toxin, and ATP plus pertussis toxin were 53–200%, 337–354%, and 445–485%, respectively (three experiments).

Effects of Mg^{2+} on the ATP-induced sensitization of adenylyl cyclase. In previous studies, submillimolar levels of free Mg^{2+} were necessary for the observation of both PMA/PKC-induced sensitization and desensitization, as well as cAMP-dependent protein kinase-mediated desensitization, in the assays of hormone-stimulated adenylyl cyclase (9, 11, 12). Low free Mg^{2+} levels were also required for observation of the ATP-induced sensitizations. When the free Mg^{2+} concentration was raised to 10 mM, the augmentations of epinephrine-, PGE_1-, and forskolin-stimulated activities were eliminated (Fig. 7).

Effect of cell density on the ATP-induced sensitization of adenylyl cyclase. In the initial attempts to study the ATP-induced sensitization of adenylyl cyclase, the response was found to be highly variable and difficult to reproduce. In subsequent experiments, we found that the effects of ATP were reduced when cells were grown to high densities. Consequently, with the exception of the data described in this section, all the experiments were performed using cells grown to preconfluency or low density. The adenylyl cyclase activity in LM5 cells grown in monolayers was most affected by high density cell growth conditions. The sensitizing effects of ATP on 1–10 μM PGE_1-stimulated adenylyl cyclase activity were reduced from 97 ± 7% (26 experiments) in preconfluent cells to 8 ± 4% (eight experiments) in cells grown to confluence. Monolayer cultures of LβAR were also affected by cell density. In these cells the ATP-induced augmentation of 1–10 μM epinephrine-stimulated adenylyl cyclase in preconfluent versus confluent cells was 76 ± 7% (28 experiments) and 21 ± 6% (eight experiments), respectively. L cells grown in suspension were least affected by cell density, but the response was significantly lower in cells grown to high densities. In L cells expressing the βAR that were grown in suspension to an average density of 0.35×10^6 cells/ml, the percentage of augmentation was 115 ± 18% (six experiments) and 147 ± 17% (six experiments) for 10 μM epinephrine- and 2 μM PGE_1-stimulated adenylyl cyclase activities, respectively. When these cells were grown to an average density of 1.0×10^6 cells/ml, 10 μM epinephrine- and 2 μM PGE_1-stimulated adenylyl cyclase activities were enhanced by 61 ± 19% (four experiments) and 65 ± 25% (four experiments), respectively.

Comparison of the effects of purinergic receptor agonists on adenylyl cyclase activity. In an attempt to characterize the type of purinoceptor involved in the sensitizing effect on adenylyl cyclase, several P_2 purinergic receptor agonists were evaluated in LβAR cells. As shown in Table 1, of all the agonists tested at 30 μM concentrations, only ATP and UTP caused significant sensitization. At concentrations of 100–300 μM, App(NH)p also caused a significant sensitization (data not shown). This rank order of potency (ATP $\geq$ UTP $\gg$ App(NH)p; ADP and ADP-β-S, no significant effect) was the same for epinephrine-stimulated adenylyl cyclase activity (data not shown). These data are consistent with those reported by Fine *et al.* (29) for the P_{2U} receptor. However, more analogues must be examined before any firm conclusions can be made.

Effects of carbachol, ATP, and PMA combinations on adenylyl cyclase activity. To gain further information concerning the pathways mediating the effects of ATP and PMA, the experiments shown in Fig. 8 were performed. LM5 cells were treated with either 30 μM ATP, which gives a maximal effect on adenylyl cyclase, or 30 μM carbachol in combination with 10 nM PMA (a concentration that gives approximately one half the maximal sensitizing effect of PMA). A slightly greater than additive augmentation with ATP and PMA and a highly significant synergistic interaction with the combination of PMA and carbachol were found. In contrast, the augmentation caused by treatment with maximal concentrations of ATP and carbachol were not significantly different than with ATP alone.

Effect of PMA-induced down-regulation of PKC on the ATP-induced sensitization of adenylyl cyclase. To investigate the possible involvement of PKC isozymes in the actions of ATP, LβAR cells were pretreated for 19 hr with 0.2 μM PMA to down-regulate PKC, and then treated for 20 min with either 0.5 μM PMA or 100 μM ATP. Sensitization of adenylyl cyclase caused by short term treatment with PMA was completely eliminated in cells pretreated for 19 hr with PMA (Fig. 9). However, the long term PMA treatment did not diminish the effect of ATP on hormone-stimulated adenylyl cyclase.

The extent of the down-regulation of PKC after the 19-hr PMA treatments was assessed by PKC activity measurements. In experiments using small-scale DE52 anion exchange chromatography to purify PKC, the average loss in PMA/Ca^{2+}/phosphatidylserine-stimulated PKC activity in the cytosolic fraction after overnight exposure to 0.2 μM PMA was 88 ± 6% (six experiments). In three additional assays carried out on peak PKC cytosolic fractions isolated using large-scale DE52 chromatography (see Materials and Methods), a 97 ± 2% (three experiments) loss of PMA/Ca^{2+}/phosphatidylserine-stimulated PKC activity was observed after overnight exposure to 0.2 μM PMA. We were unable to measure significant PKC activity in the particulate fractions in any of these experiments. This is perhaps a function of the excess EGTA in our cell lysis buffers, which would predictably cause most of the PKC to be found in our cytosolic fractions.

Discussion

We have determined that ATP, acting through stimulation of a P_2 purinergic receptor, causes a rapid and prolonged 50–150% sensitization of epinephrine-, PGE_1-, and forskolin-induced stimulations of adenylyl cyclase in L cells. The sensitization is stable, surviving purification of membranes on sucrose gradients, consistent with a covalent modification or the persistence of a positive regulator.

In contrast to the effects of ATP, treatment of the LM5 clone with the muscarinic agonist carbachol does not result in a significant sensitization of adenylyl cyclase. The lack of an effect with carbachol treatment is surprising, in light of the fact that, relative to ATP, carbachol produces 5–10-fold higher levels of inositol phosphates, a greater initial spike of Ca^{2+} release, and a more prolonged elevation of intracellular free Ca^{2+} (23). These data indicate that it is unlikely that the inositol 1,4,5-trisphosphate/Ca^{2+} branch of the PIP_2 hydrolysis pathway is involved in the sensitization, and they do not support the simplistic prediction that the hormonal activation of PIP_2 hydrolysis, which results in the production of DAGs, will inevitably lead to the sensitization of adenylyl cyclase. It must be taken into consideration, however, that carbachol was able to potentiate synergistically the sensitization caused by low concentrations of PMA (Fig. 8). Our data do not provide an explanation for this effect, but it is possible that the increased Ca^{2+} levels caused by carbachol may decrease the EC_{50} for PMA-induced activation of PKCs. In support of this, the *in vitro* studies of Bell and co-workers (30) suggest that Ca^{2+}, phosphatidylserine, and DAG cooperatively activate PKC.

Is there a role for PKCs in the sensitization of adenylyl cyclase by ATP? Because PMA sensitization is mediated via PKCs, which are accepted to be the only known receptor for the phorbol esters, it is of use to compare the actions of PMA and ATP on adenylyl cyclase. First, both ATP and PMA cause a stable augmentation of hormone- and forskolin-stimulated adenylyl cyclase, consistent with a phosphorylation of some component of the adenylyl cyclase system downstream from the receptors. Second, observation of the sensitization by both agents requires the use of low Mg^{2+} in the adenylyl cyclase assays, and high levels completely obscure the effect. Third, measurement of the maximal sensitization of epinephrine- or forskolin-stimulated adenylyl cyclase requires 10 μM GTP. Fourth, both agents cause the partial loss of G_i-mediated inhibition of adenylyl cyclase. Consistent with this, blockade of G_i action by pertussis toxin treatment reduces the augmentation caused by either ATP or PMA, at least in terms of the fold stimulation over the actions of pertussis toxin alone. Finally, both PMA and ATP increase forskolin as well as hormonal stimulations of adenylyl cyclase. These data are consistent with PKC mediation of the ATP sensitization.

However, other data suggest that the PKCs involved in the ATP-induced sensitization may be different from those activated by PMA. First, the effects of PMA are different; that is, PMA causes a 2-fold increase in the K_{act} for hormonal stimulation of adenylyl cyclase, whereas ATP appears to cause only a 10–20% decrease in the K_{act}. This may reflect activation by PMA of PKC isozymes that are not activated by ATP. Second, down-regulation of PKCs by PMA eliminates the sensitization caused by short term treatment with PMA but has no significant effect on the ATP-induced augmentation. This suggests that the PKCs involved in the PMA augmentation of adenylyl cyclase are not involved in that caused by ATP. However, this conclusion must be tempered by the possibility that the chronic PMA treatment could cause other effects. For example, Hepler *et al.* (31) demonstrated in rat liver WB cells that long term PMA treatment caused a potentiation of angiotensin II-stim-

ulated PLC activity. From their observations, these authors concluded that the down-regulation of PKC by long term PMA treatment resulted in the loss of a PKC-mediated desensitization of the angiotensin II receptor-stimulated response.

Consistent with our results, recent studies indicate that the effects of hormonal activation of the Ca^{2+}/PLC pathway on cellular processes are often quite different from one another and from those of phorbol esters. For example, it has been shown by Issandou and Rozengurt (13) that bradykinin induces a far more transient activation of PKC in Swiss 3T3 cells than either vasopressin or bombesin. In general, activation and translocation of PKC by hormones that stimulate PLC or by the addition of DAGs are usually considerably less than those caused by phorbol esters. The work of Kiley *et al.* (32) demonstrates that phorbol dibutyrate and thyrotropin-releasing hormone cause markedly different down-regulation of PKCs in GH_4C_1 cells. Thus, it is possible that there is an isozyme of PKC involved in mediating the ATP sensitization that either does not bind active phorbol esters (33) or is not down-regulated (33). Further, whereas activity measurements of the PMA/Ca^{2+}/phosphatidylserine-stimulated PKC demonstrated a >85% down-regulation, the remaining 10–15% of PKC activity could be 100% of a putative PKC isozyme involved in the ATP-induced sensitization. It should be noted the characteristics of *in vivo* activation of PKCs may be quite different from those of PKCs in cell-free assays when substrates such as histones are utilized. Our current methods of detection may not be sensitive enough to identify isozymes of PKC that exist in relatively low abundance but that may play central roles in the biochemical processes.

Several scenarios can explain the sensitization of adenylyl cyclase by ATP and carbachol involving PKC isozymes. One explanation for the differences observed in the carbachol- and ATP-induced effects on adenylyl cyclase may be their activation of different phospholipases, such as PLC and PLD. These phospholipases may utilize substrates other than PIP_2, such as phosphatidylcholine or phosphatidylethanolamine. Because there are differences in the substrates and products of PLC or PLD hydrolysis, different species of DAGs can be produced, which in turn could lead to the activation of select isozymes of PKC (34). There is convincing evidence that both muscarinic and P_2 purinergic receptors activate phosphatidylcholine PLC or PLD in certain cell types (34, 35). Thus, it is possible that either carbachol or ATP activates one or both of these lipases in L cells, resulting in selective activation of PKCs.

Measurement of the molecular species of DAGs produced and the activity of specific PKC isozymes after carbachol and ATP treatments are needed, and these studies are currently in progress. Knowledge of these factors may be a prerequisite for determination of the mechanism(s) involved in the sensitization of adenylyl cyclase caused by activation of P_2 purinergic and M5 muscarinic receptors in L cells.

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