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Endothelin- and ATP-Induced Inhibition of Adenylyl Cyclase Activity in C_6 Glioma Cells: Role of G_i and Calcium

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SUMMARY

Endothelin-1 (ET) and ATP mobilize Ca^{2+} in rat C_6 glioma cells by stimulating phosphoinositide turnover. Both agents also inhibit adenylyl cyclase (AC) activity in C_6 glioma cells. The goal of this study was to characterize the molecular mechanisms responsible for the inhibition of AC activity. The administration of either ET, ATP, A23187, or thapsigargin to cells simultaneously with isoproterenol for 5 min inhibited isoproterenol-stimulated cAMP synthesis by a maximum of 60%, 91%, 65%, and 68%, respectively. Pretreatment of cells with pertussis toxin (PTX) did not alter the inhibitory effects of A23187 or thapsigargin, whereas the inhibitory effects of ET or ATP were completely eliminated. Removal of extracellular Ca^{2+} and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester treatment failed to affect the inhibition caused by ET or ATP, whereas the inhibition caused by A23187 or thapsigargin was completely eliminated in Ca^{2+}-free medium and was attenuated by 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester treatment. The inhibition by both receptor agonists in the earlier phase (30 sec) of the AC reaction was, however, reduced by using either Ca^{2+}-free medium or PTX pretreatment. The administration of 3-isobutyl-1-methylxanthine or Ro 20–1724 suggested that the inhibitory effects of A23187 and thapsigargin were partially due to Ca^{2+}-dependent stimulation of PDE activity. Short term treatment with phorbol-12-myristate-13-acetate (PMA) had no effect on isoproterenol-stimulated AC activity. However, the inhibition of cAMP induced by ET or ATP, but not by A23187 or thapsigargin, was diminished by PMA, suggesting that the receptor signal via G_i was blocked by PMA treatment. The antagonistic effect of PMA was blocked by staurosporine. All four agents still inhibited AC activity in cells that had been treated with PMA for 24 hr to deplete protein kinase C. ET produced an additional decrease in AC activity in cells that had been treated with a maximally effective concentration of A23187 or thapsigargin. The ET- or ATP-induced decrease in cAMP levels showed homologous desensitization. These results demonstrate that ET_A receptors and ATP receptors in C_6 glioma cells inhibit AC activity primarily by interaction with a PTX-sensitive G_i and partially by elevation of $[Ca^{2+}]_i$. Protein kinase C activation is not responsible for agonist-induced inhibition of AC but appears to uncouple the G_i/AC system activated by ET or ATP.

ET and ATP mobilize intracellular Ca^{2+} in a variety of cell types. The major mechanism of action for ET and ATP is the stimulation of PI turnover with the production of two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol. However, activation of other intracellular signal transduction pathways can accompany the stimulation of PI hydrolysis by both agents in some cell types. For example, ET can either increase (1, 2) or decrease (3–9) AC activity. ATP binding to ATP receptors is coupled to the inhibition of AC activity (10–13). We have shown that ET and ATP act on ET_A and ATP receptors, respectively, in C_6 glioma cells, producing a robust increase in PI turnover (14, 15). ET and ATP also increase $[Ca^{2+}]_i$ in C_6 glioma cells by mobilizing the intracellular Ca^{2+} pool via the generation of inositol-1,4,5-trisphosphate and by activating Ca^{2+} influx through receptor-operated Ca^{2+} channels (14, 15). Both ET and ATP have also been reported to inhibit isoproterenol- and forskolin-stimulated cAMP synthesis in C_6 glioma cells (4, 13). The inhibition of AC caused by ET has an absolute requirement for extracellular Ca^{2+} (4); however, that caused by ATP seems to involve a PTX-sensitive mechanism (13).

There are several possible mechanisms for the receptor-mediated changes in cAMP levels. The receptor could be directly, independently of Ca^{2+} fluxes, coupled to AC-stimulatory (G_s) or -inhibitory (G_i) GTP-binding proteins, which cause an increase or a decrease in AC activity, respectively. Many recep-

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ABBREVIATIONS: ET, endothelin-1; AC, adenylyl cyclase; PMA, phorbol-12-myristate-13-acetate; PTX, pertussis toxin; PDE, phosphodiesterase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PI, phosphoinositide; PLC, phospholipase C; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PKC, protein kinase C; G_i, inhibitory GTP-binding regulatory protein; G_s, stimulatory GTP-binding regulatory protein; G_p, GTP-binding protein linked to phospholipase C; PSS, physiological saline solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tor-mediated changes in intracellular cAMP levels, such as those caused by carbachol (16), bradykinin (17), ATP (17), and epinephrine (18), have recently been attributed to effects on $[Ca^{2+}]_i$. The accumulating evidence, therefore, suggests the existence of complex "cross-talk" between the PI and cAMP signaling pathways (18–20). The strongest evidence for the involvement of intracellular Ca^{2+} in the AC system is that increases in $[Ca^{2+}]_i$ could either increase (21, 22) or decrease (23, 24) cAMP formation, depending on the cell type and the stimulation state of AC. On the other hand, the reason for suspecting that PKC is involved in the AC system is that activators of PKC could either potentiate (25–27) or inhibit (28–30) AC activity, depending on the cell type, the agonist used, and the regimen for the PKC activator. The results in FRTL-5 thyroid cells and diencephalic glia have suggested that the inhibitory effect of ET on cAMP production is in part mediated by a PKC-dependent pathway (6, 7).

Because ET and ATP can robustly activate at least two signal transduction pathways, i.e., one via increased $[Ca^{2+}]_i$ and a second via diacylglycerol/PKC, we investigated whether both signal transduction pathways mediated the ET- and ATP-induced inhibition of AC activity in C_6 glioma cells, by comparing the pharmacological profiles of A23187 (a Ca^{2+} ionophore), thapsigargin, and activators of PKC. Thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, can initially release intracellular Ca^{2+} without concomitant PI breakdown or PKC activation and, by an unknown mechanism, continuously activate plasma membrane Ca^{2+} entry, which is subsequent to the depletion of the intracellular Ca^{2+} pool (31–33). We also examined the contribution of Ca^{2+}/calmodulin-dependent PDE to cAMP levels with the PDE inhibitors IBMX and Ro 20–1724. We now report that ET and ATP inhibit cAMP accumulation predominantly by direct actions via G_i on AC and partially by elevation of $[Ca^{2+}]_i$.

Experimental Procedures

Materials. ET was obtained from Peptide Institute, Inc. (Osaka, Japan). Thapsigargin and octylindolactam V were purchased from Biomol (Plymouth Meeting, PA). Ro 20–1724 was obtained from Research Biochemical Inc. (Natick, MA). ATP, A23187, IBMX, PTX, (−)-isoproterenol, PMA, staurosporine, forskolin, and EGTA were products of Sigma Chemical Co. (St. Louis, MO). BAPTA/AM was from Molecular Probes (Eugene, OR). cAMP assay kits were from Amersham (Arlington Heights, IL).

Cell culture. Rat C_6 glioma cells with an original passage number of 39 (American Type Culture Collection, Rockville, MD) were grown at 37° in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, under 5% CO_2. Cells with additional passage numbers of 13–35 were subcultured in 35-mm Petri dishes.

AC activity assay. Routinely, AC activity was determined by measuring isoproterenol-stimulated cAMP accumulation. When the cells reached confluence, they were washed with PSS and incubated for 20 min at 37°. The PSS contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 20 mM HEPES, pH 7.4, unless otherwise indicated. The reaction was initiated by the addition of 10 μM isoproterenol with or without ET, ATP, A23187, or thapsigargin. Unless indicated otherwise, the reaction was incubated at 37° for 5 min and then terminated by aspiration of the reaction mixture and addition of 0.4 ml of 0.1 N HCl. The dishes were incubated at 37° for 30 min. The cells were scraped into Eppendorff tubes with an additional 0.2 ml of 0.1 N HCl, and the suspensions were centrifuged. The supernatant was neutralized with 10 N NaOH and assayed for cAMP levels with the Amersham [^{3}H]cAMP assay kit. Cell pellets were dissolved with 0.1 N NaOH and assayed for protein content by the method of Lowry *et al.* (34), using bovine serum albumin as the standard. The content of cAMP was expressed as pmol/mg of protein. All measurements in each experiment were made in triplicate.

Results

Effects of drugs on isoproterenol-stimulated cAMP synthesis. C_6 glioma cells stimulated with 10 μM (−)-isoproterenol for 5 min dramastically increased cAMP synthesis from 32.7 ± 3.3 to 4992 ± 226 pmol/mg of protein (16 experiments). ET (100 nM), ATP (100 μM), A23187 (30 μM), thapsigargin (3 μM), PMA (100 nM), and octylindolactam V (100 nM) by themselves had no significant effect on the basal cAMP content (data not shown). The simultaneous addition of isoproterenol with either ET, ATP, A23187, or thapsigargin, at the concentrations indicated above, to the cells caused a marked inhibition of isoproterenol-elicited cAMP synthesis, by about 60 ± 2% (16 experiments), 91 ± 1% (13 experiments), 59 ± 4% (nine experiments), and 64 ± 3% (11 experiments), respectively (Fig. 1). However, pretreatment of cells with PMA (100 nM) or octylindolactam V (100 nM) for 10 min had no effect on the cAMP synthesis caused by isoproterenol (Fig. 1). Dose-dependent inhibition by ET of isoproterenol-stimulated cAMP synthesis was observed for a concentration range of 1–100 nM, with an EC_{50} value close to 4.5 nM, and maximal inhibition occurred at 30 nM (Fig. 2A). ET-3, another ET isopeptide, also inhibited isoproterenol-stimulated cAMP formation, with efficacy comparable to that of ET, but its inhibition was less potent (EC_{50}, 200 nM). Isoproterenol-stimulated cAMP accumulation was time-dependently increased within 10 min, and ET-induced inhibition was observed within this same period (Fig. 2B). ATP, in a concentration range of 3–300 μM, also inhibited the isoproterenol-stimulated cAMP synthesis in a dose-dependent manner, with an EC_{50} value of 7.1 μM and maximal inhibition at 30 μM (Fig. 3). In subsequent experiments, the concentrations of agonists used were 100 nM for ET, 100 μM for ATP, 30 μM for

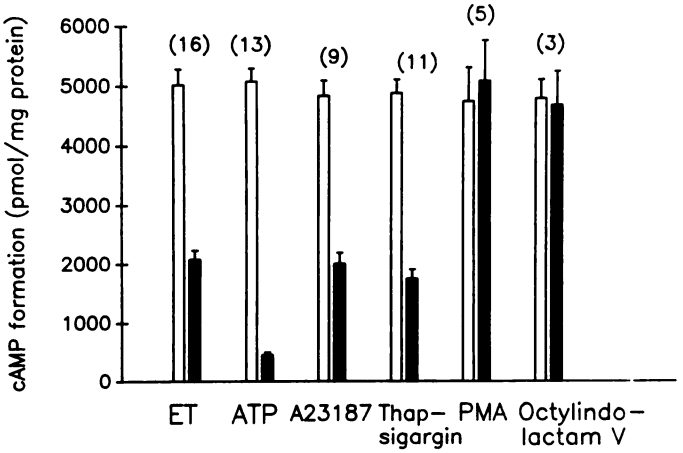

Fig. 1. Effects of drugs on isoproterenol-elicited cAMP synthesis. Cells were stimulated with 10 μM isoproterenol for 5 min in the absence (□) or presence (■) of ET (100 nM), ATP (100 μM), A23187 (30 μM), thapsigargin (3 μM), PMA (100 nM), or octylindolactam V (100 nM). PMA and octylindolactam V were added to the cells 10 min before the administration of isoproterenol. ET, ATP, A23187, and thapsigargin were added to the cells simultaneously with isoproterenol for 5 min. *Numbers in parentheses*, number of experiments; each experiment was performed in triplicate. The data are the mean ± standard error.

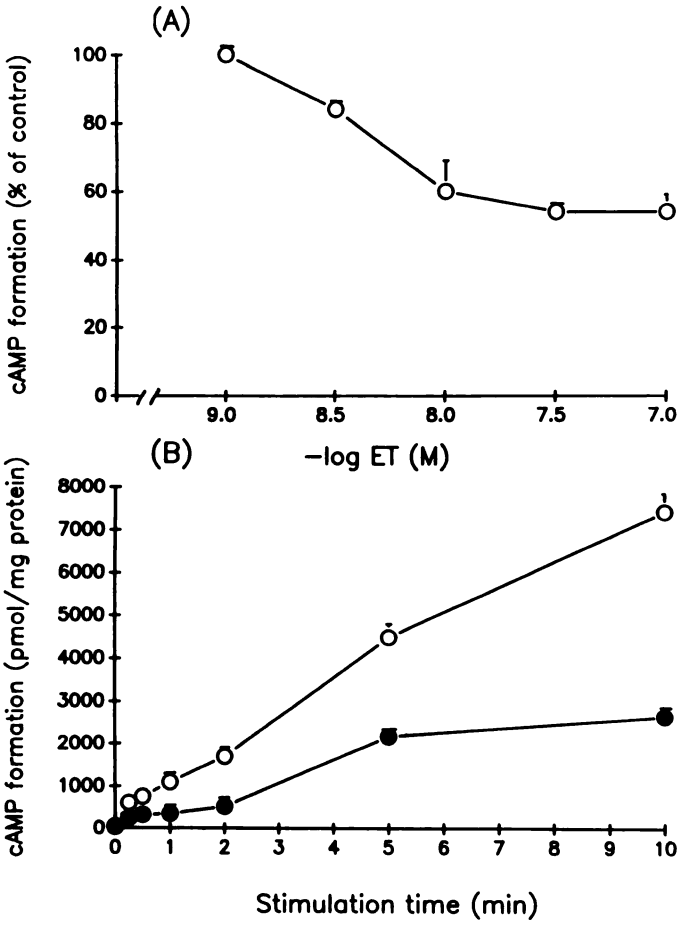

Fig. 2. Concentration- and time-dependent inhibition by ET of isoproterenol-elicited cAMP synthesis. A, Cells were treated for 5 min with 10 μM isoproterenol in the absence or presence of various concentrations of ET. The data are expressed as the percentage of control response to isoproterenol alone. B, Cells were stimulated with either 10 μM isoproterenol (○) or 10 μM isoproterenol plus 100 nM ET (●). At the indicated times, the reaction was stopped and cAMP content was determined. The basal cAMP value did not change with time. The data are the mean ± standard error from three independent experiments, which were performed in triplicate.

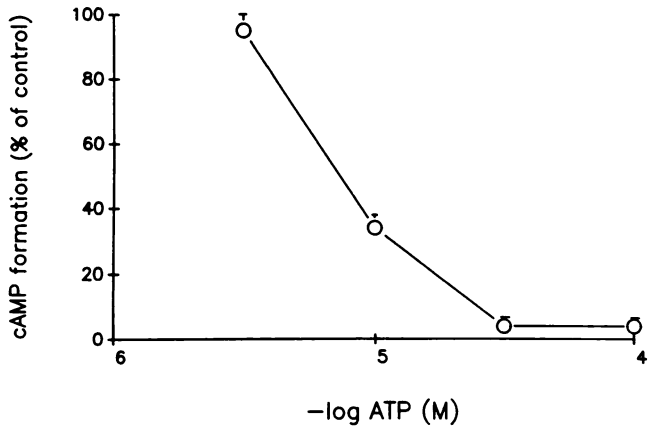

Fig. 3. Concentration-dependent inhibition by ATP of isoproterenol-elicited cAMP synthesis. The experimental design was as described in the legend to Fig. 2A. The data are mean ± standard error from three independent experiments, which were performed in triplicate.

A23187, and 3 μM for thapsigargin. ET and ATP not only reduced the stimulation of AC by isoproterenol but also caused a significant reduction in the AC response to forskolin, an agent that directly activates the catalytic subunit of AC. Forskolin (10 μM) increased cAMP levels to 1496 ± 149 pmol/mg of protein after a 5-min stimulation. ET (100 nM) and ATP (100 μM) reduced the stimulation of AC by forskolin by 52 ± 6% (three experiments) and 71 ± 6% (three experiments), respectivley.

Effects of PTX pretreatment. Cells were pretreated with 300 ng/ml PTX for 24 hr to inactivate G_i. Isoproterenol-stimulated levels of AC activity were similar in untreated and PTX-treated cells. In a 5-min AC activity assay, PTX pretreatment completely abolished the ATP-induced inhibition and markedly reduced the ET-induced inhibition from 56 ± 2% to 19 ± 4%; however, the inhibition caused by A23187 and thapsigargin was unaffected (Fig. 4). The antagonism by PTX of ET- and ATP-induced inhibition of AC activity was also examined in a reaction that was allowed to proceed for 30 sec instead of 5 min. Simultaneous addition of ET or ATP with isoproterenol also inhibited the β-adrenergic agonist-elicited cAMP synthesis (696 ± 80 pmol/mg of protein) within 30 sec (Fig. 5). PTX pretreatment for 24 hr antagonized the inhibition caused by ET and ATP less effectively at 30 sec than at 5 min. The inhibition caused by ET was attenuated from 57 ± 3% to 38 ± 2% and that caused by ATP was attenuated from 88 ± 2% to 39 ± 8% by PTX pretreatment.

Extracellular Ca^{2+} dependence of cAMP synthesis inhibition. One of the major effects of ET and ATP in C_6 glioma cells is a rapid elevation of $[Ca^{2+}]_i$, which consists of a transient phase peaking at 30 sec (14, 15) followed by a sustained plateau. Therefore, the Ca^{2+} dependence of ET- and ATP-induced inhibition of isoproterenol-stimulated cAMP synthesis was examined and compared with the responses to A23187 and thapsigargin. In Ca^{2+}-free medium containing 1 mM EGTA, isoproterenol-stimulated cAMP synthesis within 30 sec or 5 min was reduced to approximately 75% of that in normal PSS. Under 5-min assay conditions, ET and ATP inhibited isoproterenol-

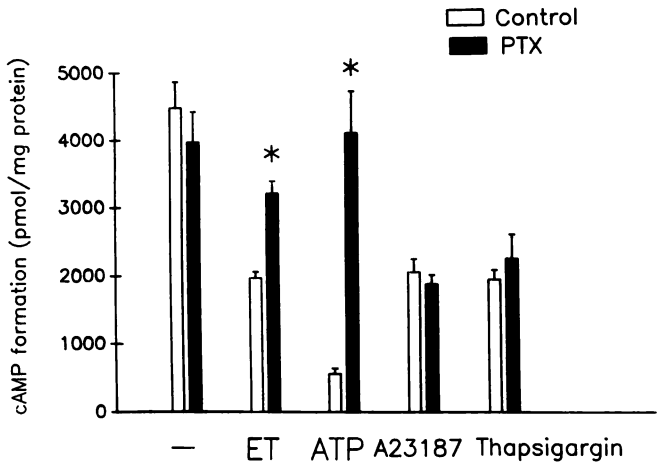

Fig. 4. Effects of PTX on ET-, ATP-, A23187-, and thapsigargin-induced inhibition of cAMP formation. Cells were pretreated with either the vehicle (*Control*) or PTX (300 ng/ml) for 24 hr before stimulation for 5 min with 10 μM isoproterenol either in the absence or in the presence of ET (100 nM), ATP (100 μM), A23187 (30 μM), or thapsigargin (3 μM). The data are the mean ± standard error from three independent experiments, which were performed in triplicate. —, No drug treatment. *, $p < 0.05$, compared with inhibition in the absence of PTX treatment.

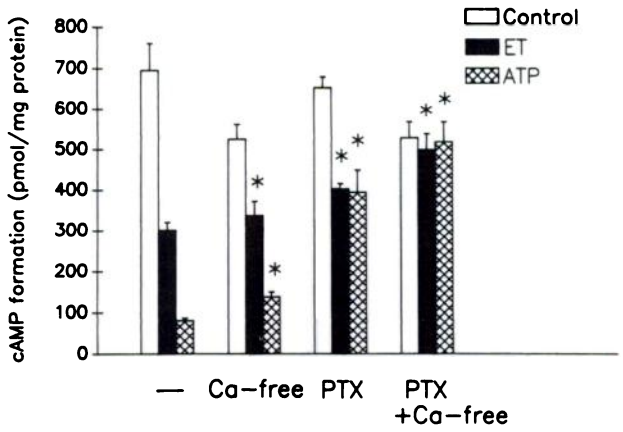

Fig. 5. Effects of Ca^{2+}-free medium and PTX on ET- and ATP-induced inhibition of cAMP formation. In the Ca^{2+}-free experiments, cells were incubated in Ca^{2+}-free medium containing 1 mM EGTA and then 10 μM isoproterenol alone or together with ET (100 nM) or ATP (100 μM) was added for 30 sec. In PTX pretreatment experiments, cells were pretreated with PTX (300 ng/ml) for 24 hr before stimulation with 10 μM isoproterenol for 30 sec in the absence or presence of ET (100 nM) or ATP (100 μM). The data are the mean ± standard error from three independent experiments, which were performed in triplicate. *, $p < 0.05$, compared with inhibition in normal PSS.

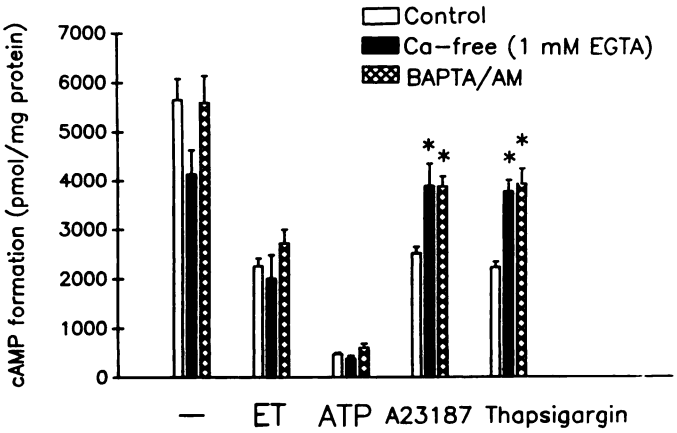

Fig. 6. Effects of Ca^{2+}-free medium and BAPTA on ET-, ATP-, A23187-, and thapsigargin-induced inhibition of cAMP formation. In the Ca^{2+}-free experiments, cells were incubated in Ca^{2+}-free medium containing 1 mM EGTA and then 10 μM isoproterenol was added for 5 min. In BAPTA/AM experiments, cells were pretreated with 100 μM BAPTA/AM for 10 min. The data are the mean ± standard error from four to six independent experiments, which were performed in triplicate. *, $p < 0.05$, compared with the inhibitory response in normal PSS.

stimulated AC activity to an extent that was not significantly different from that in normal PSS (61 ± 5% and 52 ± 12% inhibition for ET and 91 ± 1% and 90 ± 1% inhibition for ATP in normal PSS and Ca^{2+}-free PSS, respectively). However, the inhibition caused by A23187 or thapsigargin was abolished (Fig. 6). Furthermore, chelation of intracellular free Ca^{2+} with a cell-permeant Ca^{2+} chelator, BAPTA/AM, significantly attenuated the inhibitory effects of A23187 and thapsigargin but did not affect ET- and ATP-induced inhibition (Fig. 6).

In contrast, the rapid inhibition of AC activity that occurred within 30 sec after ET or ATP addition was slightly but significantly reduced in Ca^{2+}-free medium (57 ± 3% and 36 ± 7% inhibition for ET and 88 ± 2% and 74 ± 2% inhibition for ATP in normal PSS and Ca^{2+}-free medium, respectively) (Fig. 5). The combined treatment with extracellular Ca^{2+} depletion and PTX pre-exposure completely abolished the ET- or ATP-induced rapid inhibition of AC activity (Fig. 5).

Effects of activators and inhibitor of PKC. Although 100 nM PMA by itself did not affect isoproterenol-stimulated cAMP synthesis, it antagonized the inhibitory responses to ET and ATP after a 10-min pretreatment. However, A23187- and thapsigargin-induced cAMP synthesis inhibition were unchanged after PMA pretreatment (Fig. 7). Another PKC activator, octylindolactam V (100 nM), also antagonized the ET-induced inhibition of cAMP synthesis (data not shown). Long term pretreatment with PMA (100 nM) for 24 hr, however, did not alter the percentage of inhibition caused by ET, ATP, A23187, or thapsigargin (data not shown).

To shed more light on the role of PKC in antagonizing agonist-induced inhibition of cAMP accumulation, we studied the effect of staurosporine (a potent PKC inhibitor). Staurosporine alone had no significant effect on ET- or ATP-induced cAMP synthesis inhibition (data not shown). Staurosporine (1 μM) pretreatment for 10 min, however, completely reversed the antagonistic effect of PMA on ET- or ATP-induced inhibition of cAMP synthesis (Fig. 7).

Effects of inhibitors of PDE. In normal PSS containing either IBMX (1 mM) or Ro 20–1724 (500 μM), potent inhibitors of PDE, the 5-min cAMP-stimulatory response to isoproterenol was increased by 50 ± 7% or 60 ± 12% (three experiments), respectively. ET and ATP inhibited the isoproterenol-stimulated AC activity to similar extents both in the absence and in the presence of IBMX or Ro 20–1724 (Fig. 8). However, the extents of inhibition for A23187 and thapsigargin were significantly reduced in the presence of IBMX or Ro 20–1724 (Fig. 8). The decrease in the percentage of inhibition was from 72% to 45% or 54% for A23187 and from 66% to 40% or 46% for thapsigargin in the presence of IBMX or Ro 20–1724, respectively.

Additive inhibition and homologous desensitization. At maximally effective concentrations, combined treatment with ET and either A23187 or thapsigargin produced additional

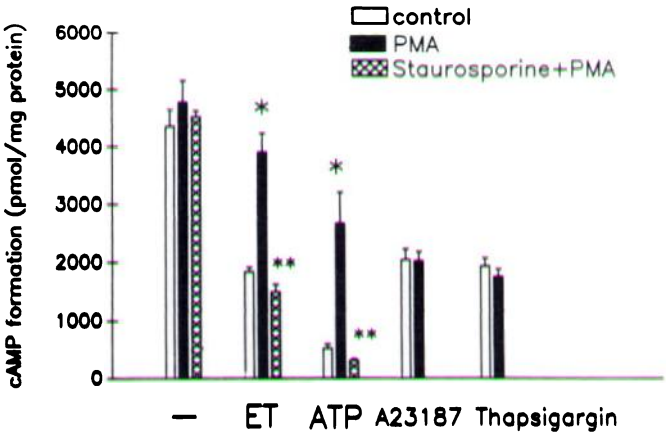

Fig. 7. Effects of PMA and staurosporine on ET-, ATP-, A23187-, and thapsigargin-induced inhibition of cAMP formation. In one group, cells were treated with 100 nM PMA for 10 min before the administration of isoproterenol simultaneously with ET, ATP, A23187, or thapsigargin for 5 min. In the other group, cells were pretreated with 1 μM staurosporine for 10 min before the addition of PMA. The data are the mean ± standard error from at least four independent experiments, which were performed in triplicate. *, $p < 0.05$, compared with the inhibitory response in the absence of PMA treatment; **, $p < 0.05$, compared with the response in the presence of PMA but in the absence of staurosporine treatment.

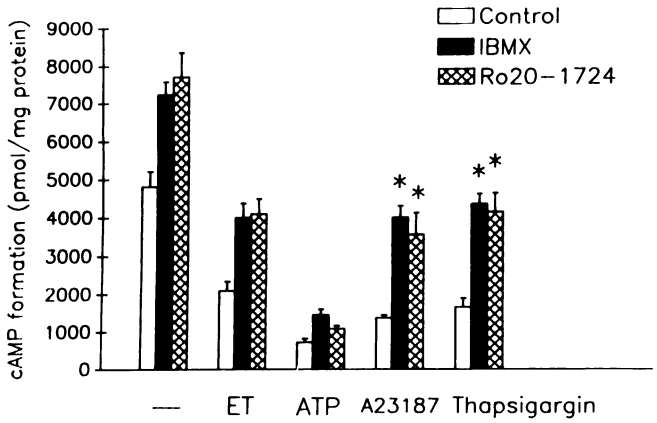

Fig. 8. Effects of IBMX and Ro 20–1724 on ET-, ATP-, A23187-, and thapsigargin-induced inhibition of cAMP formation. Cells were incubated for 20 min in PSS containing IBMX (1 mM) or Ro 20–1724 (500 μM), and then 10 μM isoproterenol was added in the absence or presence of the indicated drugs for 5 min. The data are the mean ± standard error from three independent experiments, which were performed in triplicate. *, $p < 0.05$, compared with the percentage of inhibition without IBMX and Ro 20–1724 treatment.

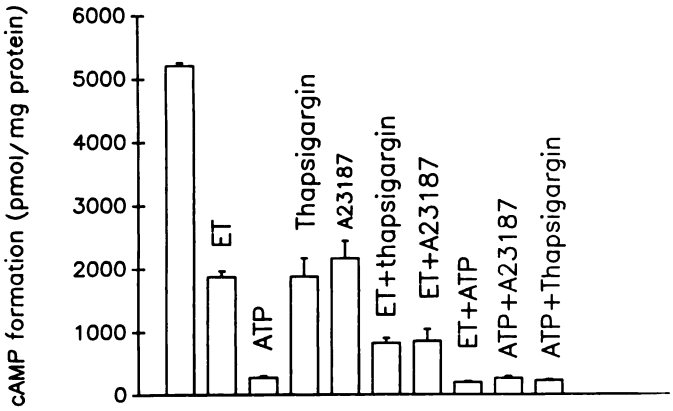

Fig. 9. Additivity of the inhibition of AC produced by maximally effective concentrations of ET (100 nM), ATP (100 μM), A23187 (30 μM), and thapsigargin (3 μM). AC activity was measured in the presence of 10 μM isoproterenol and the other indicated additions for 5 min. The data are the mean ± standard error from three triplicate experiments.

inhibition of isoproterenol-stimulated cAMP accumulation (Fig. 9). In fact, similar percentages of inhibition by ET were observed in untreated, A23187-treated, and thapsigargin-treated cells (63%, 60% and 57%, respectively). Because ATP caused approximately 90% inhibition of cAMP synthesis, no additional statistically significant inhibition was observed after combining ATP with either ET, A23187, or thapsigargin (Fig. 9).

Homologous desensitization of receptors for ET and ATP is shown in Table 1. Prior exposure of cells to 100 nM ET for 30 min attenuated the inhibition of isoproterenol-induced cAMP accumulation elicited by subsequent ET administration; however, the inhibitory effects of ATP, A23187, and thapsigargin were unchanged. Similar homologous desensitization was observed when cells were exposed to 100 μM ATP for 1 hr.

Discussion

The present study defined some of the properties of ET- and ATP-induced inhibition of AC activity in C_6 glioma cells that may be relevant to understanding the molecular mechanisms involved. Thus far, three mechanisms have been suggested to mediate the cAMP-lowering effect of ET in different cell types, i.e., PTX-sensitive G_i (5, 6, 8, 9), Ca^{2+} influx (4, 6), and a PKC-dependent pathway (6, 7). Our present results demonstrate that the inhibitory action of ET and ATP on AC is primarily due to the direct coupling of receptors to G_i, whereas the inhibition secondary to an increase in $[Ca^{2+}]_i$ plays only a minor role. The cAMP-lowering effects of ET and ATP were sensitive to PTX, suggesting that the PTX substrate G_i is involved in the inhibitory effects of both agonists. This conclusion is further supported by our preliminary data indicating that isoproterenol-stimulated AC activity in isolated plasma membranes of C_6 glioma cells is also inhibited by ET in a PTX-sensitive manner.

$[Ca^{2+}]_i$ can regulate cAMP synthesis by direct stimulation (21, 35) or inhibition (17, 23, 24) of AC activity or can regulate cAMP degradation by activation of a Ca^{2+}/calmodulin-dependent PDE (36, 37). Because both ET and ATP can elicit a rapid and sustained increase in $[Ca^{2+}]_i$ in C_6 glioma cells (14, 15), we have determined the involvement of the $[Ca^{2+}]_i$ rise in ET- and ATP-induced cAMP synthesis inhibition in the early (30 sec) and late (5 min) phases of the AC reaction. These effects of ET and ATP were then compared with those of the Ca^{2+} ionophore A23187 and the non-phorbol ester tumor promoter thapsigargin. Thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, can initially release intracellular Ca^{2+} without concomitant PI breakdown or PKC activation and, by an unknown mechanism, continuously activate plasma membrane Ca^{2+} entry, which is subsequent to the depletion of the intracellular Ca^{2+} pool (31–33).

Several lines of evidence support the idea that ET and ATP inhibit C_6 AC activity at 5 min via a Ca^{2+}-independent mechanism that is different from that for A23187 and thapsigargin. First, A23187- and thapsigargin-induced inhibition of cAMP accumulation was abolished in the absence of extracellular Ca^{2+} and diminished by the treatment of cells with the intracellular Ca^{2+} chelator BAPTA. Thus, the inhibition of cAMP synthesis by both agents was due to increased $[Ca^{2+}]_i$ via extracellular Ca^{2+} influx. The large reduction of the inhibition by thapsigargin in Ca^{2+}-free medium suggests that the transient release of intracellular Ca^{2+} by thapsigargin was not sufficient to significantly inhibit cAMP accumulation. The fact that BAPTA pretreatment did not fully eliminate the A23187- and thapsigargin-induced inhibition of cAMP accumulation could be explained by the incomplete buffering of the induced $[Ca^{2+}]_i$ rise. On the other hand, the inhibitory effects of both ET and ATP were still observed in Ca^{2+}-free medium and BAPTA/AM-treated cells. Our results showing that inhibition by ET at 5 min is independent of extracellular Ca^{2+} levels is contrary to the report of Couraud *et al.* (4), who used a 10-min incubation with the same cell line, but consistent with the results from mouse striatal astrocytes (5). Second, a 24-hr pretreatment with PTX and a 10-min pre-exposure to PMA antagonized the inhibitory effects of ET or ATP but not those of A23187 or thapsigargin. Third, the maximal inhibition by ET was almost additive with that produced by A23187 or thapsigargin, suggesting that divergent mechanisms were involved. Fourth, the inhibition by ET or ATP was unchanged in the presence of IBMX or Ro 20–1724, suggesting that inhibition of cAMP accumulation induced by both receptor agonists was due to the suppression of the generation, rather than acceleration of the

TABLE 1

Homologous desensitization of ET- and ATP-induced inhibition of cAMP formation

Cells were pretreated with the vehicle, ET (100 nM, 30 min), or ATP (100 μM, 1 hr) and then washed before rechallenge with various stimuli for 5 min. Data presented are the mean ± standard error from a typical triplicate experiment, which was repeated three times with similar results. Data in parentheses indicate the percentage of the control response produced by isoproterenol alone.

Stimulus	cAMP accumulation		
	Vehicle pretreatment	ET pretreatment	ATP pretreatment
	pmol/mg of protein		
Isoproterenol	4231 ± 205 (100%)	4150 ± 107 (100%)	4579 ± 371 (100%)
Isoproterenol + ET	1732 ± 199 (41%)	3122 ± 35* (75%)	2061 ± 75 (45%)
Isoproterenol + ATP	450 ± 48 (11%)	627 ± 91 (15%)	1532 ± 221* (33%)
Isoproterenol + A23187	1396 ± 68 (33%)	1469 ± 131 (35%)	1786 ± 150 (39%)
Isoproterenol + thapsigargin	1269 ± 60 (30%)	1403 ± 28 (34%)	1740 ± 254 (38%)

* $p < 0.05$, compared with the response without ET or ATP pretreatment.

degradation, of cAMP. On the other hand, IBMX and Ro 20–1724 partially reduced the ability of A23187 and thapsigargin to inhibit the isoproterenol-provoked cAMP accumulation. The incomplete inhibition by these two PDE blockers of A23187 and thapsigargin responses suggests the existence of a Ca^{2+}-dependent but PDE-independent inhibitory pathway for AC in C_6 cells. In this context, calcium has been found to act as a competitive inhibitor of the Mg^{2+} activation of AC and as a noncompetitive inhibitor with respect to $MgATP^{2-}$, the substrate of the enzyme (38). Our results also showed that PDE, which is normally active in cells, can exacerbate or make more profound the actions of drugs that decrease cAMP levels.

Interestingly, the rapid increase in $[Ca^{2+}]_i$ elicited by ET and ATP appears to contribute to the early phase of the inhibition of cAMP accumulation in these cells. This conclusion was based on the finding that the rapid inhibition of cAMP formation seen at 30 sec was partially attenuated in Ca^{2+}-free medium. In support of this possibility, bradykinin and ATP, which stimulated PLC activity in intact NCB-20 cells, transiently inhibited cAMP accumulation in parallel with their transient mobilization of intracellular Ca^{2+} (17). In addition to the Ca^{2+}-dependent mechanism for ET- and ATP-induced rapid inhibition of cAMP formation, direct negative coupling via G_i is involved in this event, because of the partial inhibition by PTX pretreatment of the early response to ET and ATP. Thus, unlike the late response to agonists, two signaling pathways (i.e., G_i- and PLC/Ca^{2+}-dependent inhibition of AC) participate in the rapid effects of both agonists. The loss of Ca^{2+} dependence for the late response may be explained by the decay of the agonist-elevated increase in $[Ca^{2+}]_i$, as reported previously (14, 15).

Our reasons for concluding that PKC is not involved in ET- and ATP-mediated cAMP synthesis inhibition are as follows. A 10-min pretreatment of C_6 glioma cells with the PKC activators PMA or octylindolactam V, at concentrations as high as 100 nM, did not affect the isoproterenol-stimulated AC activity. These results appear to be paradoxical, because it was reported that phorbol esters can inhibit the isoproterenol-induced cAMP system in C_6 glioma cells (28, 39, 40). It should be noted that, in previous studies, cells were exposed to phorbol esters for a much longer period (1–24 hr) before the challenge with isoproterenol (28, 39, 40). In addition, ET and ATP could reproduce their AC-inhibitory responses in PKC-depleted cells, although their PI-stimulatory responses were inhibited in C_6 cells in which PKC had been depleted by long term (24-hr) phorbol ester treatment, as reported previously (39). Recently, Debernardi *et al.* (41) also reported that increases in $[Ca^{2+}]_i$ without activation of PKC suffice and are responsible for the inhibition of cAMP accumulation in C_6-2B cells (41). Furthermore, staurosporine (a potent PKC inhibitor) cannot antagonize the cAMP synthesis inhibition by ET and ATP. Unexpectedly, PMA was found to antagonize the inhibitory response to ET or ATP but not that to A23187 or thapsigargin in C_6 glioma cells. This antagonistic effect of PMA on the response to ET and ATP was abolished by staurosporine. Octylindolactam V (100 nM, 10 min), another PKC activator, also antagonized the effect of ET. The mechanism underlying the selective blockade by PMA of receptor-induced inhibition of AC in C_6 glioma might be similar to that reported in platelets and hepatocytes, where PKC activation can stabilize the inactivated form of G_i by phosphorylating the α subunit of G_i (25, 27, 42).

From this and previous studies, we concluded that ET and ATP receptors in C_6 glioma cells are coupled not only to G_i, leading to AC inhibition, but also to G_p, leading to PLC activation. The inhibition of cAMP synthesis by ET and ATP occurred within the concentration ranges that enhanced PI turnover and $[Ca^{2+}]_i$ rise (14, 15). In addition, the multiple signal transduction pathways, including PI turnover (14, 32), $[Ca^{2+}]_i$ elevation (14), and cAMP synthesis inhibition (present results), activated by ET and ATP showed homologous desensitization. Because ET has a higher potency than does ET-3 in inducing PI hydrolysis (14), $[Ca^{2+}]_i$ elevation (14), and cAMP synthesis inhibition (present results) by a PTX-sensitive mechanism in C_6 cells, we suggest that ET_A receptors are coupled to two signal transduction systems, i.e., cAMP and PI turnover systems, via a PTX-sensitive G_i and a PTX-sensitive G_p, respectively. On the other hand, PTX treatment of C_6 glioma cells suppressed the ATP-induced inhibition of cAMP formation completely but produced no inhibition of ATP-stimulated PI turnover (15). In contrast to the effects of ATP, adenosine did not induce inhibition of cAMP formation (13) or stimulation of PI turnover (15) in C_6 glioma cells, indicating that C_6 glioma cells are devoid of A_1 or A_2 receptors. Therefore, there may be two types of P_2 purinoceptors in C_6 glioma cells, as postulated by Okajima *et al.* for rat hepatocytes (10) and FRTL-5 cells (12); the P_{2p} receptor is linked to PLC via a PTX-insensitive G_p and the P_{2i} receptor is linked to AC via a PTX-sensitive G_i. A proposed model for the interrelationship between ET_A and ATP receptor-mediated AC inhibition and PI activation is shown in Fig. 10.

In summary, our results suggest that in C_6 glioma cells the cAMP synthesis-inhibitory effects of ET and ATP are mediated by direct coupling to G_i and an increase in $[Ca^{2+}]_i$ and are not

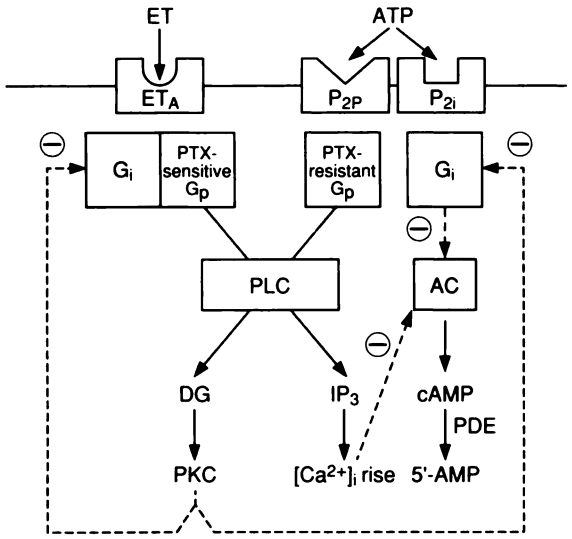

Fig. 10. Schematic representation of ET receptor- and ATP receptor-mediated inhibition of AC and activation of PLC. Activation of the ET_A receptor results in inhibition of AC activity through coupling to G_i and an increase in $[Ca^{2+}]_i$, as well as stimulation of PLC activity through coupling to a PTX-sensitive G_p. Similarly, ATP receptor activation produces AC inhibition through coupling to G_i and an increase in $[Ca^{2+}]_i$ but elicits its PLC activation through a PTX-insensitive G_p. PKC activation abolishes the inhibitory effects mediated by ET_A and P_{2i} receptors, possibly due to uncoupling by phosphorylation of the α_i subunit of G_i. *DG*, diacylglycerol; *IP_3*, inositol-1,4,5-trisphosphate.

dependent on the activation of PKC. The inhibition induced by A23187 or thapsigargin is due to Ca^{2+}-dependent inhibition of AC and activation of PDE. PKC activation appears to block ET or ATP receptor-mediated inhibition of AC activity through G_i.

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