0026-895X/86/050455-06$02.00/0

MOLECULAR PHARMACOLOGY, 29:455-460

Identification of the Phosphodiesterase Regulated by Muscarinic Cholinergic Receptors of 1321N1 Human Astrocytoma Cells

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Received August 3, 1985; Accepted February 12, 1986

SUMMARY

Agonist occupation of muscarinic cholinergic receptors of 1321N1 human astrocytoma cells results in an activation of phosphodiesterase and a resultant 50–75% attenuation of isoproterenol-stimulated cyclic AMP accumulation. The effects of a series of phosphodiesterase inhibitors on muscarinic receptor-mediated inhibition of cyclic AMP accumulation and on the activities of partially purified, soluble phosphodiesterase have been compared to determine which form of phosphodiesterase activity is regulated by muscarinic receptors. The phosphodiesterase inhibitors (50 μM) 1-methyl-3-isobutylxanthine (MIX), 1-methyl-3-isobutyl-7-benzylxanthine (7-BzMIX), 1-methyl-3-isobutyl-8-methoxymethylxanthine (8-MeOMeMIX), and 2-*O*-propoxyphenyl-8-azapurin-6-one (MB 22948) blocked the effect of muscarinic receptor activation. However, 1-isoamyl-3-isobutylxanthine (IIX) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) did not block muscarinic receptor-mediated effects but enhanced isoproterenol-stimulated cyclic AMP accumulation. Three forms of soluble phosphodiesterase activity were resolved by DEAE-cellulose chromatography and sucrose density gradient centrifugation. A calmodulin-stimulated phosphodiesterase activity was inhibited by MIX, 7-BzMIX, 8-MeOMeMIX, and MB 22948 (IC_{50} values = 1–10 μM) but was not inhibited by IIX and Ro 20-1724. The similar relative capacities of the phosphodiesterase inhibitors for blocking both the muscarinic receptor-mediated attenuation of cyclic AMP accumulation and the calmodulin-stimulated phosphodiesterase activity *in vitro* suggest that it is this form of enzyme that is regulated by muscarinic receptor stimulation.

Activation of muscarinic cholinergic receptors results in a reduction of cyclic AMP levels in a variety of tissues. This receptor-mediated decrease in intracellular cyclic AMP levels has been thought to occur primarily as a consequence of inhibition of adenylate cyclase (1–3). However, activation of cyclic nucleotide phosphodiesterase activity accounts for the muscarinic receptor-mediated attenuation of cyclic AMP accumulation in 1321N1 human astrocytoma cells (4) and dog thyroid (5). Activation of phosphodiesterase activity and subsequent decreased intracellular cyclic AMP levels may be one part of a sequalae of biochemical responses initiated by muscarinic receptor stimulation of inositol 1,4,5-trisphosphate formation and intracellular Ca^{2+} mobilization.

Multiple forms of cyclic nucleotide phosphodiesterase have been described, differing in substrate specificity, kinetics, distribution, and regulation (6, 7). Well characterized forms include a Ca^{2+}, calmodulin-stimulated cyclic nucleotide phosphodiesterase, a cyclic GMP-activated cyclic AMP phosphodiesterase, and a high affinity cyclic AMP phosphodiesterase. The purpose of the present study was to determine which form of phosphodiesterase is regulated by muscarinic receptors in 1321N1 cells. Comparison of the effects of a series of phosphodiesterase inhibitors on muscarinic receptor-mediated inhibition of cyclic AMP accumulation in intact cells to their effects on partially purified soluble phosphodiesterase activities suggests that it is a calmodulin-stimulated phosphodiesterase that is regulated by muscarinic cholinergic receptors.

Experimental Procedures

Materials. MIX and cyclic AMP were purchased from Sigma Chemical Co. (St. Louis, MO). Ro 20-1724 was a gift from Hoffman LaRoche (Nutley, NJ). MB 22948 was obtained from May & Backer (Dagenham, England). 7-BzMIX, IIX, and 8-MeOMeMIX were synthesized according to published procedures (8, 9). AG 1-X8 anion exchange resins (200–400 mesh, formate form and chloride form) were purchased from

This work was supported by United States Public Health Service Grants GM 29536, GM 21220, and HL 19325.

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ABBREVIATIONS: MIX, 1-methyl-3-isobutylxanthine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; MB 22948, 2-*O*-propoxyphenyl-8-azapurin-6-one; 7-BzMIX, 1-methyl-3-isobutyl-7-benzylxanthine; IIX, 1-isoamyl-3-isobutylxanthine; 8-MeOMeMIX, 1-methyl-3-isobutyl-8-methoxymethylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid; ISO, (−)-isoproterenol (+)-bitartrate.

Bio-Rad Laboratories (Richmond, CA). [2,8-^{3}H]Adenine (25–50 Ci/mmol) and [2,8-^{3}H]cyclic AMP (20 Ci/mmol) were purchased from ICN (Irvine, CA). [2,8-^{3}H]Cyclic AMP was further purified on AG 1-X8 before use. Crude preparations of rat brain calmodulin were prepared by the method of Teo *et al.* (10). All other materials were obtained from sources previously cited (4).

Measurement of cyclic AMP accumulation in intact cells. Human astrocytoma cells 1321N1 were maintained in culture as previously described (4). A modification (4) of the method of Shimizu *et al.* (11) was used to measure intracellular cyclic AMP levels. Separation of [^{3}H]ATP and [^{3}H]cyclic AMP was by a modification (4) of the method of Salomon *et al.* (12). Column recovery was assessed by a spectrophotometric determination (259 nm) of the cyclic AMP concentration in an aliquot of the [^{3}H]cyclic AMP eluate. Recoveries ranged from 50 to 75%.

DEAE-cellulose chromatography. Confluent culture dishes of 1321N1 cells [50–80 (150-mm) plates, 1–2 × 10^9 cells] were washed and incubated on ice with 10 mM Tris (pH 7.5), 10 mM EDTA for 15 min. The cells were harvested with a rubber policeman followed by homogenization using a Polytron (setting 6, 15 sec) or Dounce homogenizer. The homogenate was centrifuged at 40,000 × *g* for 15 min, and the resulting supernatant was centrifuged a second time at 40,000 × *g* for 15 min. The supernatant fraction (150–300 mg of protein) was applied to a column of DEAE-cellulose (2.6 × 10 cm) previously equilibrated with 1 mM sodium acetate (pH 6.5), 1 mM EGTA. The column was washed with 3–5 bed volumes of equilibration buffer, and then phosphodiesterase activity eluted with a linear gradient (160 ml) of 1–500 mM sodium acetate (pH 6.5) containing 1 mM EGTA; 2-ml fractions were collected.

Sucrose density gradient centrifugation. DEAE-cellulose fractions were pooled (see figure legends) and concentrated by ultrafiltration (Amicon CF 25), and the resulting concentrated enzyme pool was layered onto linear 5–20% (w/v) sucrose gradients containing 40 mM Tris, 5 mM mercaptoethanol, and 10 mM $MgCl_2$ (pH 8.0). Protein standards (aldolase, ovalbumin, and cytochrome *c*) were centrifuged through parallel gradients for determination of sedimentation coefficients. Samples were centrifuged under the conditions described in the figure legends.

Cyclic AMP phosphodiesterase assay. Cyclic AMP phosphodiesterase activity was assayed by a modification (13) of the procedure of Thompson and Appleman (14). A 0.4-ml reaction mixture containing enzyme, [^{3}H]cyclic AMP (150,000–200,000 cpm/assay), cyclic AMP (0.25 μM or as indicated), 5 mM $MgCl_2$, and 4 mM mercaptoethanol in 40 mM Tris (pH 7.4) was incubated for 10–30 min at 30°. EGTA, calcium chloride, and/or calmodulin were added to the reaction mixture as noted in the figure legends. Enzyme activity was stopped by immersing the tubes in a boiling water bath for 1 min. The samples were cooled on ice and then incubated at 30° for 15 min with 50 μg of snake venom (*Ophiophagus hannah*). One ml of a 1:4 slurry of Dowex AG 1-X8 resin in 3 mM acetic acid was added; the samples were incubated at 4°, centrifuged at 3,500 × *g* for 10 min, and a 0.5-ml aliquot of the supernatant fraction was counted. Buffer blanks constituted 1–2% of the total radioactivity added.

Results

Isoproterenol-stimulated cyclic AMP accumulation in intact 1321N1 astrocytoma cells was inhibited (50–75%) by the muscarinic receptor agonist, carbachol, in the absence of a phosphodiesterase inhibitor (Fig. 1). In the presence of increasing concentrations of the alkylxanthine phosphodiesterase inhibitor, MIX, the inhibitory effect of carbachol was reversed; at higher concentrations of MIX cyclic AMP accumulation was enhanced (Fig. 1, *left*). The IC_{50} for attenuation of the effect of carbachol by MIX was 18 ± 3 μM (SE, $n = 3$), whereas the IC_{50} for enhancement of ISO-stimulated cyclic AMP accumulation was 180 ± 60 μM ($n = 3$). In contrast, Ro 20-1724, an imidazolidinone derivative, increased ISO-stimulated cyclic AMP accumulation but had no effect on the capacity of carbachol to inhibit cyclic AMP accumulation (Fig. 1, *right*). Both MIX and Ro 20-1724 enhanced cyclic AMP accumulation under basal conditions, i.e., no ISO (data not shown).

These results suggested that 1321N1 astrocytoma cells contain more than one type of phosphodiesterase, with one type exhibiting sensitivity to inhibition by both MIX and Ro 20-1724, and a second type that is sensitive only to MIX and that is activated in the presence of carbachol. This hypothesis was further tested by comparing the capacity of a series of phosphodiesterase inhibitors to enhance ISO-stimulated cyclic AMP accumulation and inhibit muscarinic receptor-mediated attenuation of cyclic AMP accumulation (Fig. 2). At a concentration of 50 μM, the phosphodiesterase inhibitors MIX, 8-MeOMeMIX, and 7-BzMIX increased ISO-stimulated cyclic AMP levels and reversed the inhibitory effect of carbachol. In fact, cyclic AMP levels in the presence of these phosphodiesterase inhibitors were elevated by carbachol. MB 22948 had no significant effect on ISO-stimulated cyclic AMP levels, but partially ($n = 1$) or completely ($n = 2$) reversed muscarinic receptor-mediated inhibition of cyclic AMP accumulation. Finally, IIX and Ro 20-1724 enhanced ISO-stimulated cyclic AMP levels, both in the presence and absence of carbachol. However, carbachol still markedly inhibited cyclic AMP accumulation in the presence of IIX and Ro 20-1724, respectively, suggesting that these two compounds were incapable of blocking the phosphodiesterase activated as a consequence of muscarinic receptor stimulation.

Experiments were conducted to determine whether the differential effects of the phosphodiesterase inhibitors observed using intact 1321N1 cells could be correlated with selective inhibition of the *in vitro* activity of resolved forms of phosphodiesterase. Only the soluble forms of phosphodiesterase were studied since 85–90% of homogenate phosphodiesterase activity (assayed at 1 μM cyclic AMP) was attributable to soluble, rather than particulate, phosphodiesterase activity (data not shown). In addition, carbachol pretreatment of intact cells, homogenates, or washed particulate fractions had no apparent effect on membrane-associated or soluble phosphodiesterase activity measured *in vitro* (data not shown).

Two major peaks of soluble phosphodiesterase activity were resolved by DEAE-cellulose chromatography (Fig. 3). Further separation of the enzyme forms was achieved by centrifugation of peak I and peak II enzymes (Fig. 3) through linear sucrose density gradients (Figs. 4 and 5). The first peak of enzyme activity eluting from DEAE-cellulose had the properties characteristic of Ca^{2+}, calmodulin-sensitive phosphodiesterase. Enzyme activity was stimulated by Ca^{2+} alone and was further stimulated by maximally effective concentrations of calmodulin (Fig. 3). Stimulation by Ca^{2+} alone suggests that, although elution buffer contained 1 mM EGTA, calmodulin co-eluted with the phosphodiesterase activity. The calmodulin antagonists W-7, 48-80, and calmidazolium inhibited the Ca^{2+}-stimulated phosphodiesterase activity of peak I but did not affect basal phosphodiesterase activity (data not shown). Peak I phosphodiesterase activity migrated on sucrose density gradients as a single 6.8 S activity that was stimulated 8-fold by Ca^{2+} and a maximally effective concentration of calmodulin (Fig. 4). Lineweaver-Burk and Eadie-Hofstee kinetic plots of cyclic AMP hydrolysis were linear (data not shown), and computer-aided,

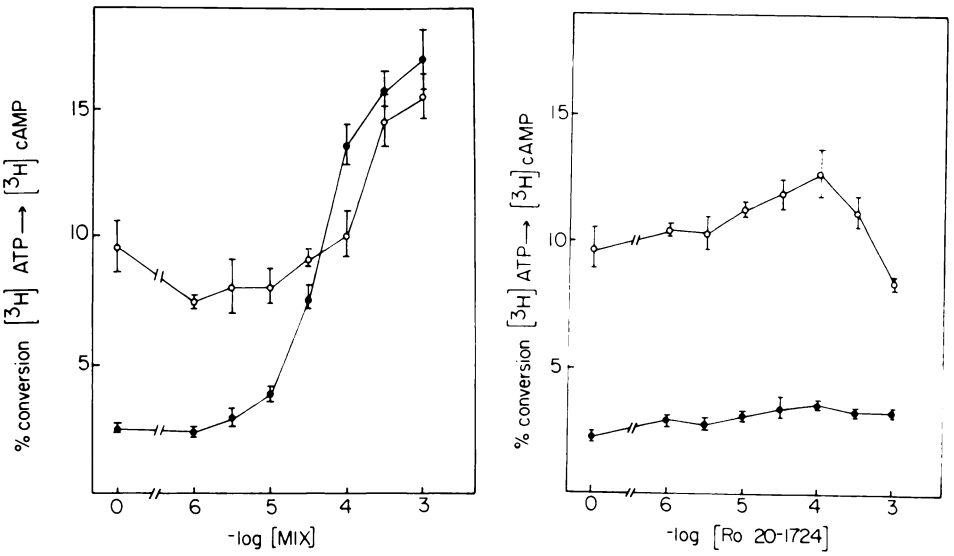

Fig. 1. Effect of MIX and Ro 20-1724 on cellular cyclic AMP levels. Cyclic AMP levels were measured as described in Experimental Procedures. The 1321N1 cells were incubated for 10 min with vehicle (1% dimethylsulfoxide) or with MIX (*left*) or with Ro 20-1724 (*right*). Cells were then challenged for 10 min with 10 μM ISO (○) or 10 μM ISO + 100 μM carbachol (●). The results shown are the average ± SD of quadruplicates and are representative of three experiments.

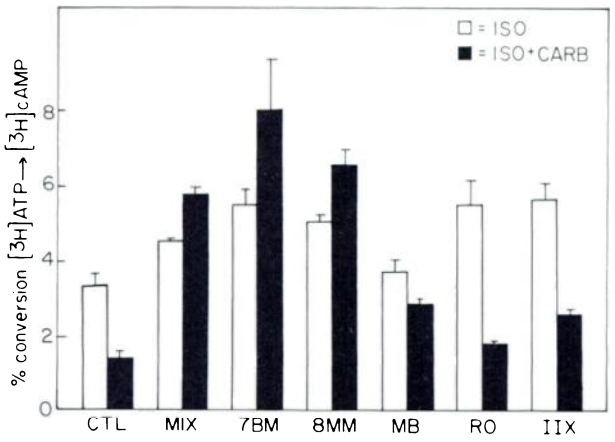

Fig. 2. Effect of phosphodiesterase inhibitors on cellular cyclic AMP levels. Cyclic AMP levels were measured as described in Experimental Procedures. The 1321N1 cells were incubated for 10 min with phosphodiesterase inhibitor or vehicle (0.25% dimethylsulfoxide). All phosphodiesterase inhibitors were made up as 20 mM stocks in dimethylsulfoxide and were diluted to a final concentration of 50 μM with Eagle's minimal essential medium-HEPES. Cells were then challenged for 10 min with ISO (10 μM; □) or ISO (10 μM) + CARB (100 μM; ■). The results shown are averages ± SD of quadruplicates and are representative of four experiments. *7BM*, 7-BzMIX; *8MM*, 8-MeOMeMIX; *MB*, MB 22948; *RO*, Ro 20-1724.

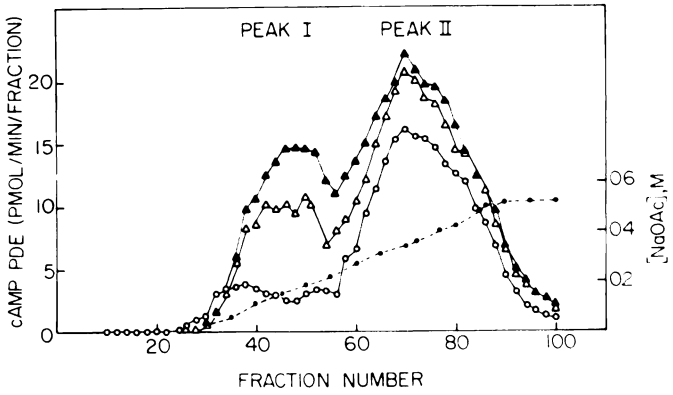

Fig. 3. Resolution of soluble phosphodiesterase activities by DEAE-cellulose chromatography. The soluble fraction from 1321N1 cells was chromatographed on DEAE-cellulose as described in Experimental Procedures. Cyclic AMP phosphodiesterase activity (0.25 μM) was assayed in the absence (○) or presence (△) of added calcium chloride (2.5 mM, final) or in the presence of Ca^{2+} plus calmodulin (▲). EGTA (250 μM) was present in all assays. The results shown are single determinations and are representative of eight preparations. In a typical experiment, fractions 36–50 were pooled as a source of peak I phosphodiesterase activity, and fractions 60–90 were pooled as a source of peak II phosphodiesterase activity.

nonlinear regression analysis gave estimated K_m values for cyclic AMP of 220 μM and 70 μM, and V_{max} values of 60 and 340 pmol/min/assay, in the presence of EGTA and calcium-calmodulin, respectively.

The second major peak of phosphodiesterase activity that eluted from DEAE-cellulose (Fig. 3, *peak II*) displayed little or no sensitivity to stimulation by calcium and calmodulin. Sucrose density centrifugation of peak II enzyme pooled from DEAE-cellulose was used to further separate enzyme fractions into two forms of activity with different apparent affinities for cyclic AMP (Fig. 5). A single but asymmetrical peak of phosphodiesterase activity, sedimenting at 3.4 S, was obtained when assayed at low substrate concentrations (0.1 μM cyclic AMP); two peaks of activity sedimenting at 3.4 S and 6.2 S were observed when activity was measured at 100-fold higher concentrations of cyclic AMP. Neither form of phosphodiesterase was activated by addition of Ca^{2+} and calmodulin, or by addition of 1 μM cyclic GMP (data not shown).

The 3.4 S peak of activity exhibited nonlinear kinetics for cyclic AMP hydrolysis; computer analysis indicated that the data were significantly better fit to a two-site model where K_m values for cyclic AMP were 0.41 and 5.6 μM, and V_{max} values were 1.8 and 5.4 pmol/min/assay, for the high and low affinity components, respectively. Based on these properties the 3.4 S enzyme form was designated as a high affinity cyclic AMP phosphosphodiesterase.

The relative potencies of phosphodiesterase inhibitors for inhibition of each of the enzyme forms resolved by anion-exchange chromatography and sucrose density gradient centrifugation were determined. MIX, 7-BzMIX, and 8-MeOMeMIX were equipotent (IC_{50} = 2 μM) for inhibiton of the Ca^{2+}, calmodulin-stimulated phosphodiesterase of peak I (Fig. 6, Table 1), whereas MB 22948 was about one-fifth as potent (IC_{50} = 10 μM). IIX and Ro 20-1724 did not inhibit the Ca^{2+},

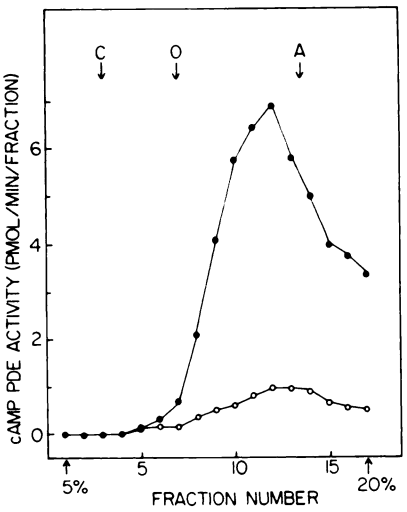

Fig. 4. Sucrose gradient centrifugation of peak I phosphodiesterase activity. Pooled fractions of peak I phosphodiesterase resolved on DEAE-cellulose were concentrated and layered onto a 3.5-ml linear 5–20% sucrose gradient. Gradients were centrifuged in an SW60 rotor at 50,000 rpm for 12 hr at 4°. Fractions were assayed for cyclic AMP phosphodiesterase activity (0.25 μM) in the absence (○) or presence (●) of added 1 mM calcium chloride plus calmodulin. The results shown are single determinations and are representative of two preparations. An average sedimentation coefficient of 6.8 S was obtained, where the individual values were 7.1 and 6.5 S. The mobility of the protein standards cytochrome *c* (*C*), ovalbumin (*O*), and aldolase (*A*) are indicated.

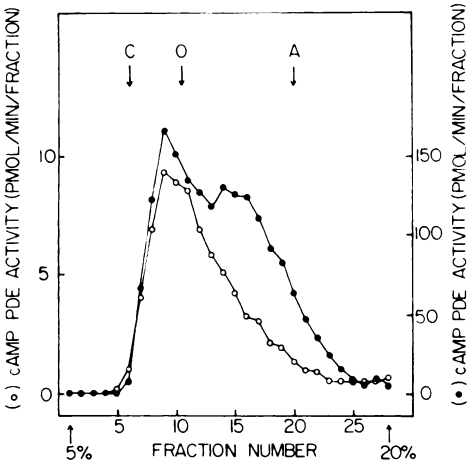

Fig. 5. Sucrose gradient centrifugation of peak II phosphodiesterase activity. Pooled fractions of peak II phosphodiesterase resolved on DEAE-cellulose were concentrated and layered onto a 10.5-ml linear 5–20% sucrose gradient. Samples were centrifuged in an SW40 rotor at 35,000 rpm for 25.5 hr at 4°. Fractions were collected and assayed for phosphodiesterase activity at 0.1 μM cyclic AMP (○) or 10 μM cyclic AMP (●). The results shown are single determinations and are representative of results obtained with two preparations. For the first peak, an average sedimentation coefficient of 3.4 S was obtained, where the individual values were 3.2 and 3.5 S. For the second peak, an average sedimentation coefficient of 6.2 S was obtained, where the individual values were 5.9 and 6.4 S. The mobility of the protein standards cytochrome *c* (*C*), ovalbumin (*O*), and aldolase (*A*) are indicated. Fractions 6–11 were pooled as a source of the peak II, 3.4 S enzyme. Fractions 13–21 were pooled as a source of the peak II, 6.2 S enzyme.

calmodulin-stimulated phosphodiesterase activity of peak I at concentrations up to 100 μM. These relative potencies of the phosphodiesterase inhibitors for blockade of Ca^{2+}, calmodulin-stimulated phosphodiesterase activity paralleled their relative

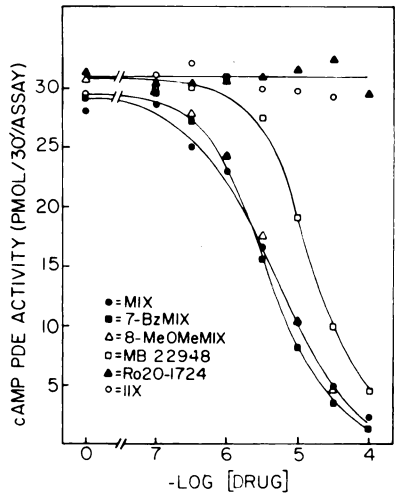

Fig. 6. Inhibition of peak I phosphodiesterase activity. Pooled fractions of peak I resolved on DEAE-cellulose were assayed for cyclic AMP phosphodiesterase activity (0.25 μM) in the presence of 1 mM calcium chloride and calmodulin. Phosphodiesterase inhibitors were made up as 20 mM stocks in dimethylsulfoxide. The final concentration of dimethylsulfoxide in all tubes was 0.5%. The results shown are averages of duplicate determinations and are representative of results obtained with three preparations.

TABLE 1

Inhibition of partially purified cyclic AMP phosphodiesterase activity

Cyclic AMP phosphodiesterase activity was assayed at a concentration of 0.25 μM cyclic AMP as described in Experimental Procedures. Peak I, 6.8 S enzyme activity was measured in the presence of 1 mM calcium chloride plus calmodulin. Pooled fractions from DEAE-cellulose (Fig. 3) were used for the source of peak I, 6.8 S enzyme, and pooled fractions from sucrose gradient centrifugation (Fig. 5) were used for the sources of peak II, 3.4 S and 6.2 S enzyme.

Drug	IC_{50} (μM)		
	Peak I, 6.8 S ($N = 3$)	Peak II, 3.4 S ($N = 2$)	Peak II, 6.2 S ($N = 1$)
MIX	2.3	4.8	1.8
7-BzMIX	2.0	8.4	2.2
8-MeOMeMIX	2.1	41	2.2
MB 22948	10	48	10
Ro 20-1724	≥100	3.3	≥100
IIX	≥32	0.9	≥32

capacities for blockade of muscarinic receptor-mediated inhibition of cyclic AMP accumulation in intact cells (Fig. 2).

In contrast to the data obtained with the Ca^{2+}, calmodulin-stimulated phosphodiesterase, IIX was the most potent inhibitor (IC_{50} = 0.9 μM) of the 3.4 S phosphodiesterase resolved by sucrose gradient centrifugation of peak II (Table 1). Ro 20-1724, 7-BzMIX, and MIX were approximately ⅕ to 1/10 as potent as (IC_{50} = 3–10 μM), and 8-MeOMeMIX and MB 22948 were approximately 50-fold less potent (IC_{50} = 40–50 μM) than IIX for inhibition of the 3.4 S phosphodiesterase.

Although the third form of phosphodiesterase (6.2 S) obtained from sucrose gradient centrifugation of peak II phosphodiesterase activity (Fig. 5) was not stimulated by Ca^{2+} and calmodulin, the profile of the relative potencies of the compounds for inhibition of this form of phosphodiesterase was similar to that obtained with peak I Ca^{2+}, calmodulin-stimulated phosphodiesterase. That is, MIX, 8-MeOMeMIX, and 7-BzMIX were potent inhibitors, MB 22948 was less potent, and Ro 20-1724 and IIX did not affect activity (Table 1).

Discussion

Results from the present study provide evidence that activation of muscarinic receptors on 1321N1 astrocytoma cells

inhibits cyclic AMP accumulation through stimulation of a Ca^{2+}, calmodulin-sensitive phosphodiesterase. The rank order of potency of a series of phosphodiesterase inhibitors for blockade of peak I Ca^{2+}, calmodulin-stimulated phosphodiesterase activity (Fig. 6, Table 1) paralleled their rank order of potency for reversal of muscarinic receptor-mediated inhibition of cyclic AMP accumulation in intact cells (Fig. 2). There was no correlation between their capacity to reverse muscarinic receptor-mediated effects on cyclic AMP accumulation and their capacity to block a high affinity cyclic AMP phosphodiesterase (peak II, 3.4 S) that was insensitive to Ca^{2+} and calmodulin (Table 1). These results are consistent with previous information regarding the muscarinic receptor system of 1321N1 cells. We have reported that the divalent cation ionophore A23187 mimics the effects of muscarinic receptor stimulation on cyclic AMP accumulation (4). Moreover, stimulation of muscarinic receptors of 1321N1 cells results in phosphoinositide breakdown and Ca^{2+} mobilization (15). A muscarinic receptor-mediated increase in the cytoplasmic Ca^{2+} concentration would result in activation of the Ca^{2+}, calmodulin-stimulated phosphodiesterase (16). This proposed sequence of events is substantiated by the demonstration of an increased activity of a similar enzyme form in intact pig coronary artery subsequent to histamine- or KCl-induced increases in cytoplasmic calcium concentration (17).

The ubiquity of this mechanism of receptor-mediated activation of phosphodiesterase activity is not known. However, Dumont *et al.* (5) have reported that activation of muscarinic receptors of dog thyroid slices results in attenuation of cyclic AMP accumulation by a mechanism involving phosphodiesterase. As with the cholinergic response of 1321N1 cells (18), pertussis toxin had no effect on the response to muscarinic receptor stimulation in thyroid slices (19), and studies with a series of phosphodiesterase inhibitors led to the conclusion that muscarinic receptor-mediated effects occurred through activation of a Ca^{2+}, calmodulin-stimulated phosphodiesterase (20). α_1-Adrenergic receptor-stimulated breakdown of phosphoinositides and Ca^{2+} mobilization in heart cells apparently is accompanied by activation of a phosphodiesterase (21). Finally, it has been reported that, in cultured rat granulosa cells, gonadotropin-releasing hormone inhibits luteinizing hormone-stimulated cyclic AMP accumulation in a Ca^{2+}-dependent manner, and the effect of gonadotropin-releasing hormone is blocked by MIX (22).

The selectivity of inhibitors for different phosphodiesterase activities formed the basis for the present study. Experiments with intact cells suggested that there are at least two forms of phosphodiesterase activity expressed by 1321N1 cells, and that the phosphodiesterase inhibitors employed in this study differ in their capacity to inhibit these forms. For example, MIX and Ro 20-1724 both increased ISO-stimulated cyclic AMP accumulation, but only MIX blocked the effects of carbachol (Figs. 1 and 2). One explanation for these results is that a form of phosphodiesterase that is inhibited both by MIX and Ro 20-1724 regulates cyclic AMP levels in the absence of muscarinic receptor agonists, i.e., in the absence of elevated cytoplasmic Ca^{2+}, and another form that is inhibited by MIX, but not by Ro 20-1724, is activated in response to cholinergic stimuli, i.e., by elevated cytoplasmic Ca^{2+}.

Three forms of soluble phosphodiesterase activity were resolved by a combination of DEAE-cellulose chromatography and sucrose gradient centrifugation. The elution profile, sedimentation coefficient, and kinetics of the peak I Ca^{2+}, calmodulin-stimulated phosphodiesterase activity were similar to those of the Ca^{2+}, calmodulin-stimulated phosphodiesterase identified in bovine brain (6, 23, 24). The rank order of potency for inhibition of this enzyme form (Table 1) was the same as that reported for the calmodulin-sensitive enzyme isolated from other tissues (5, 8, 9, 20). The 3.4 S form of phosphodiesterase exhibited characteristics of the high affinity cyclic AMP-specific phosphodiesterase, including insensitivity to Ca^{2+} or calmodulin, kinetic properties, elution profile, and sedimentation coefficient (6, 7, 25). The selective inhibition of this enzyme form by phosphodiesterase inhibitors was also consistent with previous reports (5, 8, 9, 26).

A 6.2 S form of phosphodiesterase activity also was isolated by sucrose gradient centrifugation of peak II activity (Fig. 5). The elution profile and sedimentation coefficient of the peak II, 6.2 S enzyme are similar to those of the cyclic GMP-activated phosphodiesterase of horse thyroid (27), but was not activated by 1 μM cyclic GMP. In addition, this enzyme exhibited susceptibility to phosphodiesterase inhibitors different from that of the cyclic GMP-activated phosphodiesterase of dog thyroid (20). Indeed, the relative potencies of the phosphodiesterase inhibitors for inhibition of cyclic AMP hydrolysis by this enzyme form were similar to that of the peak I Ca^{2+}, calmodulin-stimulated phosphodiesterase. It is possible that this activity may be a form of the peak I Ca^{2+}, calmodulin-stimulated phosphodiesterase that has been proteolyzed, and thus is maximally activated and insensitive to calmodulin, as has been previously described (28). However, we also cannot rule out the possibility that this is a cyclic GMP-regulated enzyme form that has been modified during isolation. Further characterization of this enzyme and the effects of muscarinic receptor agonists on intracellular cyclic GMP levels are required to eliminate unequivocally the possibility that cholinergic regulation of cyclic AMP levels involves increased synthesis of cyclic GMP.

The phosphodiesterase inhibitors MIX, 8-MeOMeMIX, and 7-BzMIX augmented ISO-stimulated cyclic AMP accumulation and blocked muscarinic receptor-mediated attenuation of cyclic AMP accumulation (Fig. 2). In fact, in the presence of these inhibitors, the combination of ISO and carbachol resulted in an elevation of cyclic AMP levels greater than that observed in the presence of ISO alone. The reason for this muscarinic receptor-mediated stimulation of cyclic AMP accumulation is not known. However, Meeker and Harden (4) have reported that, in the presence of MIX, the divalent cation ionophore A23187, in combination with ISO, stimulates cyclic AMP accumulation above that observed with ISO alone. This result, together with the more direct observation of muscarinic receptor-mediated mobilization of Ca^{2+} (15), suggests that Ca^{2+}-mediated regulation of adenylate cyclase may occur in 1321N1 cells. Such regulation could conceivably involve interactions of adenylate cyclase components with calmodulin (29) or protein kinase C (30). An increase in cyclic AMP accumulation due to musarinic receptor activation was only observed in the presence of phosphodiesterase inhibitors capable of inhibiting the Ca^{2+}, calmodulin-stimulated phosphodiesterase.

In summary, we have partially purified soluble phosphodiesterase activities from 1321N1 cells and demonstrated a strong correlation between the capacity of a phosphodiesterase inhib-

itor to inhibit Ca^{2+}, calmodulin-stimulated phosphodiesterase activity and its capacity to block muscarinic receptor-mediated inhibition of cyclic AMP accumulation in intact cells. These results suggest that the muscarinic receptor-mediated reduction of cyclic AMP levels in 1321N1 cells occurs, at least in part, through activation of a Ca^{2+}, calmodulin-sensitive phosphodiesterase.

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