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Muscarinic Cholinergic Receptor-Mediated Activation of Phosphodiesterase

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SUMMARY

A site was identified on 1321N1 human astrocytoma cells which bound [^{3}H]quinuclidinyl benzilate with high affinity (K_D = 11 pM) and in competition binding experiments exhibited agonist and antagonist affinities consistent with those of a muscarinic cholinergic receptor. Stimulation of this receptor on intact cells with 100 μM oxotremorine inhibited isoproterenol-induced accumulation of cyclic AMP by 50–60% and decreased the time to maximal levels by approximately 3-fold. Addition of 0.5 mM 3-isobutyl-1-methylxanthine to the medium prior to hormonal challenge (a) markedly increased the accumulation of cyclic AMP in the presence of either isoproterenol or isoproterenol plus oxotremorine, (b) increased the time to steady state to approximately 10 min in both cases, and (c) resulted in an apparently noncompetitive blockade of the inhibitory effects of oxotremorine on cyclic AMP accumulation. The capacity of intact cells to degrade cyclic AMP was measured by incubation for 5 min with isoproterenol or isoproterenol plus oxotremorine, followed by blockade of the synthetic reaction with a saturating concentration of propranolol. The rate constant (k_{deg}) for degradation of cyclic AMP in the absence of oxotremorine was 0.38 min^{-1}. In the presence of oxotremorine, greater than 80% of the total degradation occurred at a much more rapid rate (k_{deg} = 1.08 min^{-1}) than in cells incubated in the absence of oxotremorine. In the absence of Ca^{2+} in the medium, the inhibitory effects of oxotremorine were essentially reversed, and the k_{deg} calculated in the presence of oxotremorine was the same as the k_{deg} calculated in its absence. The divalent cation ionophore A23187 also reduced isoproterenol-stimulated cyclic AMP levels by 50–75%. The effects of A23187 could be blocked by 3-isobutyl-1-methylxanthine, suggesting that decreases in cyclic AMP levels in the presence of A23187 occur through activation of phosphodiesterase. Co-addition of A23187 and oxotremorine produced no greater reduction in cyclic AMP levels than was observed with the addition of either drug alone. Oxotremorine had no effect on adenylate cyclase activities in membrane preparations from 1321N1 cells. Taken together, these data indicate that a muscarinic cholinergic receptor on 1321N1 astrocytoma cells regulates cyclic AMP levels predominantly through a Ca^{2+}-mediated activation of phosphodiesterase. Analysis of phosphodiesterase activity in cell-free preparations indicated that the regulatory effects of muscarinic receptors on phosphodiesterase are lost upon cell lysis.

INTRODUCTION

Activation of muscarinic cholinergic receptors (1–3) as well as a number of other receptors (4–6) leads to reduction in cyclic AMP levels in a wide variety of tissues. The demonstration that adenylate cyclase activity is reduced in cell-free preparations by agonists acting at these receptors (7–12) has led to the tacit assumption that hormone-induced decreases in intracellular cyclic AMP levels mainly occur through effects on the synthetic activity of the cyclic AMP system. Such need not be the case, since the turnover rate of cyclic AMP in most cells is relatively great as a result of the activity of phosphodiesterase. Thus, receptor-mediated enhancement of phosphodiesterase activity could lead to a reduction in the accumulation of cyclic AMP. Indeed, data have been reported which indicate that muscarinic receptor-mediated activation of phosphodiesterase may account at least in part for the muscarinic receptor agonist-induced reduction of cyclic AMP levels in thyroid slices (13) and WI-38 fibroblasts (14).

Recently, we have initiated a series of studies designed to enhance understanding of the mechanisms of interaction of muscarinic cholinergic receptors with the cyclic AMP-generating system. 1321N1 Human astrocytoma cells have served as an excellent model system for study

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of the regulation of the cyclic AMP system under a wide variety of experimental paradigms (15–19). Using [^{3}H]QNB[2] we have identified a population of binding sites on 1321N1 cells that expresses the properties exhibited by muscarinic cholinergic receptors in a variety of other tissues. We have confirmed the results of Gross and Clark (19), who previously demonstrated that activation of muscarinic receptors reduces cyclic AMP levels in these cells. Evidence is provided indicating that this reduction in cyclic AMP levels occurs as a result of muscarinic receptor-mediated activation of phosphodiesterase rather than due to inhibitory effects on adenylate cyclase. A preliminary description of portions of this work has been reported (20).

METHODS

Materials. DMEM, trypsin, and fetal calf serum were purchased from GIBCO (Grand Island Biological Company, Grand Island, N. Y.). OXO sesquifumarate, (−)-ISO bitartrate, atropine sulfate, (−)-scopolamine hydrochloride, methacholine chloride, IBMX, guanosine 5′-triphosphate sodium salt, carbachol, pilocarpine HCl, Hepes, Tris, 2-mercaptoethanol, cobra venom (*Ophiophagus hannah*), and Dowex 50-X8-400 (H^+ form) were obtained from Sigma Chemical Company (St. Louis, Mo.). EDTA, EGTA, and neutral alumina (Brockman activity 1, 80–200 mesh) were obtained from Fisher Scientific Company (Pittsburgh, Pa.). A23187 was purchased from Calbiochem (San Diego, Calif.), and Dowex 1-X8 anion exchange resin (200–400 mesh, chloride form) was obtained from Bio-Rad Laboratories (Richmond, Calif.). Dimethyl sulfoxide (Fisher) was used to dissolve A23187. All chemicals and drugs were of reagent grade or the highest quality available.

[^{3}H]QNB (40.2 Ci/mmole) and [α-^{32}P]ATP (15–33 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Adenine (26–29 Ci/mmole) was obtained from Amersham (Arlington Heights, Ill.), and [2,8-^{3}H]cyclic AMP (15 Ci/mmole) was purchased from ICN (Irvine, Calif.).

Cell culture. 1321N1 Human astrocytoma cell stock cultures were maintained in 75 cm^2 plastic culture flasks (Falcon) in DMEM supplemented with 5% fetal calf serum and free of antibiotics. Cells were grown in a humidified incubator at 37° in an atmosphere of 8% CO_2, 92% air. All stock flasks were confluent and 7–10 days old at the time of subculture. Subculture was accomplished by aspirating the medium and detaching cells by the addition of 0.025% trypsin in isotonic NaCl-citrate buffer (pH 7.8). The cells were seeded into 150-mm glass plates or glass scintillation vials at a dilution of approximately 20,000 cells/ml of medium and at a plating density of 5000–8000 cells/cm^2. Medium was replenished every 4th day.

Membrane preparation. Membranes were prepared by aspirating the growth medium and swelling the cells by the addition of 10 ml of 1 mM Tris buffer, pH 7.5 at 0°. After 15 min on ice in the lysing buffer the cells were scraped from the plate with a rubber spatula. Washed membranes were prepared by three centrifugations at 30,000 × *g* for 10 min in 10 mM Tris containing 1 mM EDTA, pH 7.5 at 0°. The final pellet was resuspended in 10 mM Tris, pH 7.5 at 37°, at a concentration of 100–180 μg of protein per milliliter. Membranes not used on the day of preparation were frozen at −80° in a small aliquot of 250 mM sucrose/5 mM $MgCl_2$/50 mM Tris, pH 7.5 at 0°. Tissue stored in this manner showed no changes in [^{3}H]QNB binding over a 2-month period.

Muscarinic receptor assay. The muscarinic receptor assay was initiated by adding a membrane suspension (500–800 μg of protein in 10 mM Tris, pH 7.5 at 37°; 130–180 μg/ml) to polypropylene tubes (Sarstedt) containing test agents and [^{3}H]QNB at concentrations ranging from 5 to 400 pM. The final assay volume was 5 ml. After a 60-min incubation at 37° the reaction was terminated by the addition of 5 ml of wash buffer consisting of 10 mM Tris (pH 7.5 at 37°) and 145 mM NaCl, followed by immediate filtration through Schleicher & Schuell No. 30 glass-fiber filters. Each filter was washed twice with 5 ml of wash buffer. Nonspecific binding was defined as the amount of [^{3}H]QNB bound in the presence of 1 μM atropine and ranged from 5% to 10% of total [^{3}H]QNB retained by the filters. Specific binding was linear at concentrations up to 800 μg of added membrane protein per assay. K_i values for competing drugs were calculated according to the relationship $K_i = IC_{50}/[1 + ([^3H]QNB)/K_D]$, where IC_{50} equals the concentration of competing drug that inhibits specific [^{3}H]QNB binding by 50% and K_D equals the dissociation constant of [^{3}H]QNB (21).

Measurement of cyclic AMP accumulation in intact cells. The accumulation of cyclic AMP was measured in 1321N1 cells usually grown for 7 days in 24-mm glass scintillation vials (4.5 cm^2 surface area). At the time of assay, each vial contained approximately 0.8–1.0 × 10^6 cells, or 100–150 μg of protein. In most experiments cyclic AMP accumulation was monitored by a modification (15) of the method of Shimizu *et al.* (22). The cells were incubated in 1.0 ml of DMEM + 25 mM Hepes (pH 7.4) containing [^{3}H]adenine (2.0 μCi/ml) for 1 hr in a shaking water bath at 37°. The medium was then aspirated, the cells were washed once with 1.0 ml of DMEM-Hepes, and a fresh aliquot of 0.95 ml of DMEM-Hepes was added.

In most experiments, the synthesis of [^{3}H]cyclic AMP was induced by the addition of ISO in a volume of 50 μl (10 μM final concentration). The inhibition of [^{3}H]cyclic AMP accumulation was measured by challenging the cells with a muscarinic receptor agonist (usually OXO, 100 μM final concentration), simultaneously with the addition of ISO. In some experiments the cells were challenged by the addition of 10 μM PGE_1 in the presence or absence of 100 μM OXO. The reaction was terminated by aspiration followed immediately by the addition of 1 ml of 5% trichloroacetic acid. [^{3}H]Cyclic AMP was extracted into 5% (w/v) trichloroacetic acid overnight at 4°.

Chromatographic separation of the [^{3}H]cyclic AMP from [^{3}H]ATP was carried out according to a modification of the method of Salomon *et al.* (23). Each sample

[2] The abbreviations used are: QNB, (−)-quinuclidinyl benzilate; DMEM, Dulbecco's modified Eagle's medium; OXO, oxotremorine sesquifumarate; ISO, (−)-isoproterenol (+)-bitartrate; IBMX, 3-isobutyl-1-methylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PGE_1, prostaglandin E_1.

was transferred onto a 0.8-cm diameter plastic column (Kontes) filled to a depth of 2.5 cm with Dowex 50-X8 which had been pre-equilibrated with distilled water. [^{3}H]ATP was eluted into scintillation vials by adding 3 ml of distilled water. The Dowex column was then placed over an alumina column, and 4 ml of water were applied to the Dowex column with the [^{3}H]cyclic AMP eluting directly onto the alumina column. [^{3}H]Cyclic AMP was then eluted into scintillation vials by the addition of 2 ml of 50 mM Tris (pH 8.0) to the alumina column. Recovery of [^{3}H]cyclic AMP was 60–70% according to external [^{3}H] cyclic AMP standards run in parallel with the experimental samples. The [^{3}H]cyclic AMP formed during hormonal challenge is expressed as the percentage conversion of [^{3}H]ATP to [^{3}H]cyclic AMP, i.e., (dpm of [^{3}H] cyclic AMP/dpm of [^{3}H]ATP + dpm of [^{3}H]cyclic AMP) × 100. In some experiments endogenous cyclic AMP also was determined by radioimmunoassay, using the method of Steiner *et al.* (24). Trichloroacetic acid extracts containing [^{3}H]cyclic AMP were handled as described above except that 100-μl samples were taken from each sample for radioimmunoassay after the Dowex column step. Results obtained with the radioimmunoassay of cyclic AMP were always qualitatively similar to those obtained with the prelabeling assay. The [^{3}H]adenine prelabeling technique was routinely used because of the speed and relative reproducibility of this method.

Adenylate cyclase assay. Adenylate cyclase activity was quantitated by a modification of the method of Salomon *et al.* (23). Each assay tube contained, in final concentrations, the following reagents: 0.1 mM [α-^{32}P] ATP (60 cpm/pmole; 0.5 μCi/assay), 1.0 mM [^{3}H]cyclic AMP (30,000 cpm/assay), 8 mM creatine phosphate, creatine phosphokinase (6 units/assay), 1 μM GTP, 5 mM $MgSO_4$, 1.33 mM EGTA, 150 mM NaCl, and 25 mM Hepes (pH 7.5), in a final volume of 0.150 ml. Reactions were started by adding approximately 100 μg of membrane protein to a test tube containing all other reagents. Incubation was carried out for 30 min at 30°, and the reaction was terminated with the addition of 0.85 ml of 5% trichloroacetic acid. [α-^{32}P]ATP was separated from [^{32}P]cyclic AMP by sequential column chromatography over Dowex and neutral alumina as described above.

Cyclic AMP phosphodiesterase assay. The activity of cyclic AMP phosphodiesterase was examined by utilizing the assay procedure of Thompson and Appleman (25) as modified by Thompson *et al.* (26). The 1321N1 cells grown on 150-mm plates were washed three times with ice-cold 1 mM Tris buffer, pH 7.5. Ten milliliters of 40 mM Tris buffer (pH 8.0) were added to each plate, the cells were suspended by scraping the plate with a rubber spatula, and the tissue was homogenized with a Brinkmann Polytron (Setting 5.5 for 20 sec). The homogenate was either added directly to the assay tubes or centrifuged at 30,000 × *g* for 20 min. In the latter case, the pellet was resuspended in assay buffer and then both the supernatant and pellet were assayed immediately. Tissue was maintained at 0° throughout the preparation. Tissue samples (30–50 μg of protein/200 μl) were added to tubes containing 40 mM Tris (pH 8.0 at 30°), 20 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 60 μg of bovine serum albumin, and concentrations of cyclic AMP ranging from 0.5 to 400 μM. [^{3}H]Cyclic AMP (previously purified over an anion exchange column) was held constant at approximately 100,000 cpm/assay. The total assay volume was 0.4 ml.

After incubation at 30° for the appropriate time (5–25 min), the reaction was terminated by transferring the tubes to a water bath at 100° for 45 sec. The tubes were then placed in an ice bath, 100 μl of cobra venom (1 mg/ml) were added, and the tubes were incubated for another 10 min at 30°. The tubes were transferred to an ice bath, 1 ml of absolute methanol was added, and the resulting mixture was passed over a Dowex 1-X8 (200–400 mesh) anion exchange resin followed by 1 ml of methanol. The eluate was counted with 6 ml of scintillation cocktail at approximately 40% efficiency.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (27), using bovine serum albumin as a standard.

RESULTS

Characterization of [^{3}H]QNB binding. [^{3}H]QNB was utilized to determine whether 1321N1 astrocytoma cells exhibited binding sites with properties similar to those described for muscarinic cholinergic receptors in other tissues. Equilibrium binding was attained within 30 min at a [^{3}H]QNB concentration of 100 pM (data not shown). The association rate constant (k_1) for [^{3}H]QNB, calculated from time course experiments, was $1.2 \pm 0.4 \times 10^9$ M^{-1}/min^{-1} ($n = 3$). Dissociation of the receptor-ligand complex was induced by adding atropine (1 μM final concentration) to membranes that had been incubated with [^{3}H]QNB for 60 min (data not shown). Very slow dissociation of radioligand was observed ($t_{1/2} = 237$ min). A semilogarithmic plot of these data could be fit by a single line. The first-order rate constant for dissociation (k_2) of [^{3}H]QNB was 0.003 ± 0.0004 min^{-1} ($n = 4$). The kinetically derived dissociation constant ($K_D = k_2/k_1$) was 3.2 ± 1.1 pM.

Saturation binding isotherms for [^{3}H]QNB are depicted in Fig. 1 (*top*). Scatchard analyses (Fig 1, *bottom*) of these data suggest that [^{3}H]QNB interacts with a single high-affinity binding site on 1321N1 cells. The average K_D for [^{3}H]QNB determined with 7-day cultures was 11.1 ± 0.8 pM ($n = 3$), and the density of binding sites was 46.2 ± 5.5 fmoles/mg of protein ($n = 3$). As is also illustrated in Fig. 1, the density but not the affinity of [^{3}H]QNB binding sites varied with the age of the cultures. The specific activity of muscarinic receptors progressively increased over 12 days of culture. The density of [^{3}H]QNB binding sites was 70–80 fmoles/mg of protein on day 12. These data are in marked contrast to the expression of *beta*-adrenergic receptors in the same cultures. As we have previously reported (16, 28), the density of *beta*-adrenergic receptors in 1321N1 cells increases markedly during the first several days after subculture, then declines dramatically once confluence (i.e., day 4) of the cultures is attained.

The apparent affinities of a series of muscarinic receptor agonists and antagonists for competition at [^{3}H]QNB binding sites of 1321N1 cells were similar to those previously reported for muscarinic receptors in a variety of other tissues. K_i values calculated for scopolamine, atro-

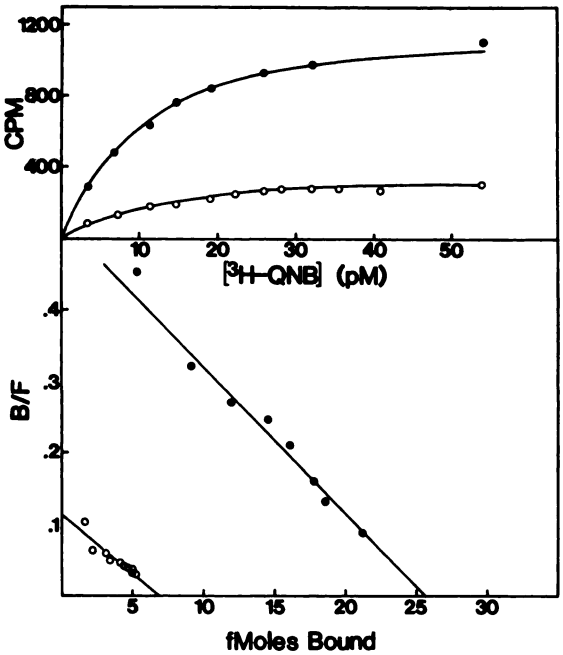

FIG. 1. *Saturation binding isotherms for [^{3}H]QNB*

Cells were harvested from 4-day (○) or 7-day (●) cultures. [^{3}H]QNB specifically bound was measured as a function of ligand concentration (2–54 pM) at a protein concentration of approximately 120 μg of protein per milliliter. The data are plotted as saturation binding curves (*top panel*) or as Scatchard plots (*bottom panel*). The data for Scatchard analyses are plotted as femtomoles of [^{3}H]QNB specifically bound (abscissa) versus bound ligand divided by the free ligand concentration (× 10^6). The results are representative of five experiments carried out in triplicate.

pine, OXO, pilocarpine, methacholine, and carbachol were 0.087 ± 0.008, 0.103 ± 0.008, 224 ± 22, 554 ± 35, 875 ± 90, and 2446 ± 464 nM, respectively (n = 3 or 4).

Inhibition of cyclic AMP accumulation in whole cells. Co-addition of OXO with agonists of the *beta*-adrenergic or prostaglandin receptors of 1321N1 cells resulted in a marked reduction in the accumulation of [^{3}H]cyclic AMP as compared with accumulation in the presence of ISO or PGE_1 alone (Table 1). Results were qualitatively the same irrespective of whether the adenine prelabeling technique or radioimmunoassay of endogenous cyclic AMP levels was used. Concentration-effect curves for inhibition of ISO-stimulated [^{3}H]cyclic AMP accumulation by OXO and carbachol are illustrated in Fig. 2. Maximal inhibition was achieved at an OXO concentration of 100 μM and a carbachol concentration of 1000 μM. The presence of 100 nM atropine resulted in an approximately 50-fold shift in the OXO concentration-effect curve to the right. The $K_{0.5}$ for OXO in four experiments was 920 ± 490 nM. The efficacy of OXO was less in 1- to 3-day cultures [25–40% inhibition of ISO-stimulated levels) than in 4- to 12-day cultures (50–60% inhibition of ISO-stimulated levels (data not shown)].

Time course of muscarinic receptor-mediated inhibition of cyclic AMP accumulation. The time course of accumulation of cyclic AMP after the simultaneous addition of ISO (10 μM) and OXO (100 μM) was examined (Fig. 3). After approximately 1 min, the presence of OXO resulted in a substantial decrease in the accumulation of

TABLE 1

Effects of OXO on cyclic AMP accumulation

Cyclic AMP accumulation was measured as described under Methods. IBMX, ISO, OXO, and PGE_1 were present at 500, 10, 100, and 10 μM, respectively. Data are presented as means ± standard error of the mean of four determinations.

Condition	% Conversion of [^{3}H]ATP to [^{3}H]cyclic AMP
Basal	0.14 ± 0.01
Basal + IBMX	0.20 ± 0.01
ISO	2.42 ± 0.24
ISO + OXO	1.41 ± 0.10
ISO + IBMX	3.89 ± 0.37
ISO + OXO + IBMX	4.16 ± 0.12
PGE_1	1.79 ± 0.12
PGE_1 + OXO	1.16 ± 0.12
PGE_1 + IBMX	2.94 ± 0.35
PGE_1 + OXO + IBMX	3.37 ± 0.21

cyclic AMP. The relative level of inhibition increased rapidly with time, eventually reaching a steady state after approximately 3 min. No further change in the relative level of inhibition was apparent for at least 20 min. The alteration with time of the degree of inhibition of cyclic AMP accumulation in cells incubated with OXO was also expressed as an alteration in the time course of approach to maximal cyclic AMP levels. The time to maximal cyclic AMP levels was approximately 4 min in the presence of ISO alone and 1–2 min in the presence of ISO plus OXO. Under the conditions of these assays little

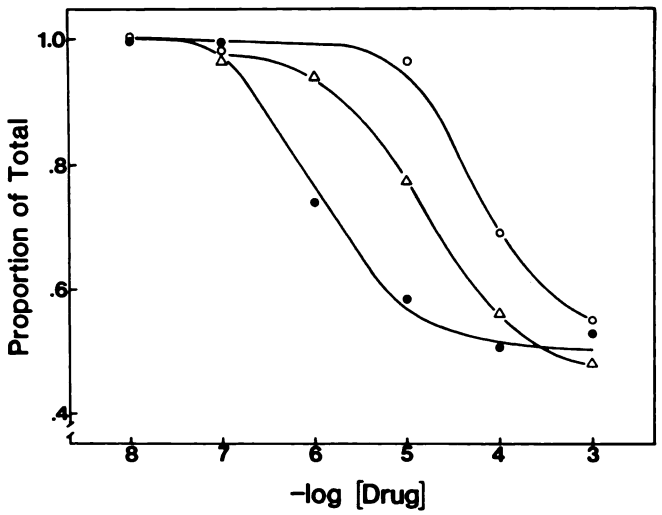

FIG. 2. *Concentration effect curves for the inhibition of [^{3}H]cyclic AMP accumulation by OXO or carbachol*

[^{3}H]Cyclic AMP accumulation was measured as described under Methods in the presence of 10 μM ISO and various concentrations of OXO (●) or carbachol (△). An OXO concentration effect curve was also generated in the presence of 100 nM atropine (○). Results are plotted as percentage of total cyclic AMP accumulation in the presence of ISO alone. The data are representative of three experiments.

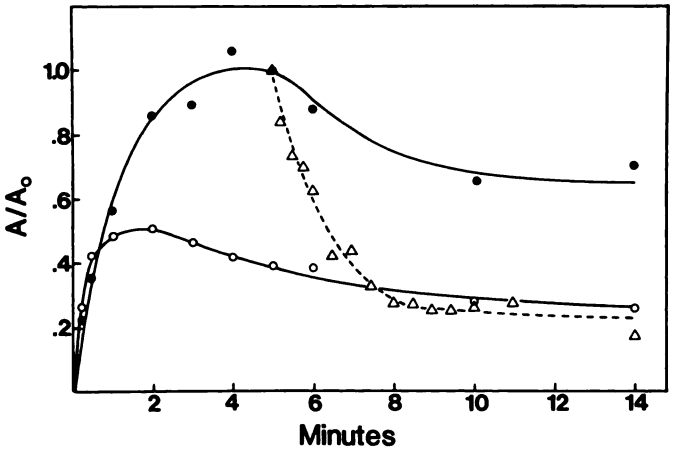

FIG. 3. *Effect of OXO on [^{3}H]cyclic AMP accumulation in the presence of ISO*

The time course of [^{3}H]cyclic AMP accumulation in 1321N1 cells was followed subsequent to the addition of 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○). OXO (100 μM) was also added to dishes after 5 min of incubation with 10 μM ISO alone, and cyclic AMP levels were followed with time (△). Each point was normalized by expressing the data as the amount of cyclic AMP accumulation (A) relative to the level of accumulation after 5 min in ISO alone (A_0). Each point represents the mean of triplicate determinations. The data are representative of five similar experiments.

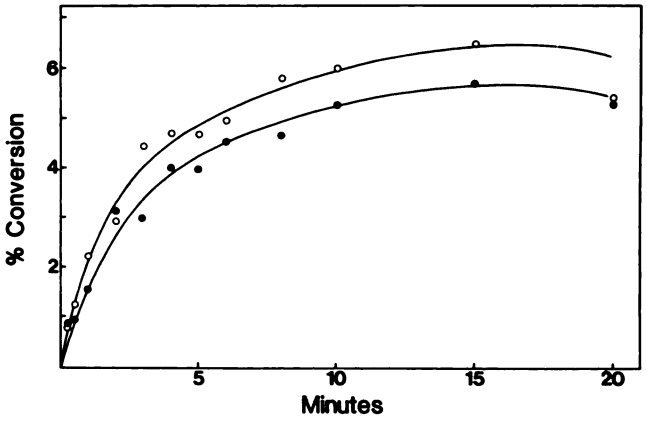

FIG. 4. *Effect of IBMX on the accumulation of [^{3}H]cyclic AMP*

Cells were challenged with 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○). Both challenges were carried out in the presence of 500 μM IBMX. Each point represents the mean of triplicate determinations. The data are representative of three similar experiments.

cyclic AMP (<5% of intracellular levels) could be detected in the medium, indicating that the observed changes in cyclic AMP accumulation do not reflect alterations in the egress of nucleotide from the cells (data not shown). A rapid desensitization to the effects of catecholamines on adenylate cyclase occurs in 1321N1 cells (15, 18). The effects of OXO on cyclic AMP accumulation in the presence of ISO potentially could be explained by an enhancement of the rate or extent of desensitization to *beta*-receptor agonists. However, this is not the case. Isoproterenol (10 μM)-stimulated adenylate cyclase activity in homogenates from 1321N1 cells previously incubated for 5 min with 1 μM ISO was 44.3 ± 2.0% of control; isoproterenol-stimulated adenylate cyclase activity from cells previously incubated with 1 μM ISO + 100 μM OXO was 41.0 ± 0.8% of control.

Addition of the phosphodiesterase inhibitor, IBMX, to the incubation medium resulted in a 2-fold enhancement in the accumulation of [^{3}H]cyclic AMP and an increase in the time to maximal accumulation to 10 min (Fig. 4). In contrast to the results obtained in the absence of a phosphodiesterase inhibitor, OXO failed to inhibit the accumulation of [^{3}H]cyclic AMP in the presence of IBMX (Fig. 4; Table 1). In addition, the time to maximal accumulation (approximately 10 min) in the presence of OXO plus ISO was the same as in cells incubated with ISO alone. The lack of effect of OXO in the presence of IBMX was not due to a direct interaction of IBMX with muscarinic receptors, since no change in the amount of [^{3}H]QNB bound or in OXO competition binding curves was observed in the presence of 0.5 mM IBMX (data not shown).

Cyclic AMP degradation in the presence of OXO. Both the decrease in the time to maximal cyclic AMP accumulation in the presence of OXO and the reversal of the inhibitory effects of OXO in the presence of IBMX suggest that muscarinic receptor activation reduces cyclic AMP accumulation by effects on the degradative, not synthetic, components of the cyclic AMP system. In order to test this possibility more directly, the kinetics of [^{3}H]cyclic AMP degradation was measured after activation of either the *beta*-adrenergic receptor or both the *beta*-adrenergic and muscarinic receptor. Subsequent to termination of [^{3}H]cyclic AMP synthesis by the addition of a saturating concentration of propranolol, [^{3}H]cyclic AMP levels decreased rapidly (Fig. 5, *top panel*). As we have reported (16), degradation of [^{3}H]cyclic AMP in 1321N1 cells previously challenged with ISO occurs in a single component by a first-order process (Fig. 5, *lower panel*). The rate constant for degradation was 0.38 ± 0.04 min^{-1} (n = 4). In contrast, the rate of loss of [^{3}H]cyclic AMP from cells incubated with OXO was 2- to 3-fold faster than in cells that were exposed only to ISO. The $t_{1/2}$ for degradation was reduced from approximately 85 sec in the absence of OXO to 35 sec in its presence. A semilogarithmic plot (Fig. 5, *lower panel*) of [^{3}H]cyclic AMP degradation in the presence of OXO was biphasic, with a rapid component (k_{deg} = 1.08 ± 0.05 min^{-1}; n = 5) accounting for greater than 80% of the loss of [^{3}H]cyclic AMP and a slower component (k_{deg} = 0.25 ± 0.06 min^{-1}) accounting for the remainder. Since in the presence of propranolol essentially no adenylate cyclase activity occurs, these data reflect only the capacity of the cells to degrade cyclic AMP. Thus, the increased rate of loss of cyclic AMP in the presence of OXO indicates that activation of muscarinic receptors reduces [^{3}H]cyclic AMP accumulation in 1321N1 cells at least in part by increasing phosphodiesterase activity. Similar results (k_{deg} = 1.01 ± 0.08 min^{-1}; n = 3) were obtained when OXO was added at the time of propranolol addition rather than at the time of ISO addition (data not shown). Under this condition, the effect of OXO on cyclic AMP degradation was measured in cells whose initial cyclic AMP levels were the same as control.

Ca^{2+} dependence of muscarinic receptor-mediated effects. In light of the apparent interaction of muscarinic receptors with phosphodiesterase, the role of Ca^{2+} in the action of muscarinic receptors was examined. In the

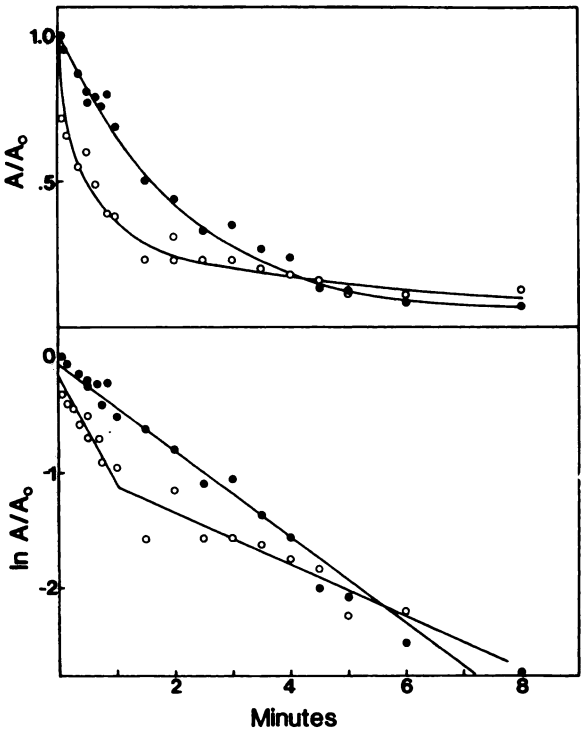

FIG. 5. *Effect of OXO on cyclic AMP degradation*

Cells were incubated in the presence of 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○). After five min, the synthetic reaction was stopped by the addition of 50 μl of propranolol to give a final concentration of 10 μM. At the indicated times, the cells were fixed by aspiration and addition of 1.0 ml of 5% trichloroacetic acid.

Top panel: [^{3}H]Cyclic AMP level (A) at the indicated times divided by the initial level (A_o) of [^{3}H]cyclic AMP at the time of propranolol addition is plotted versus time. The 5-min level of [^{3}H]cyclic AMP accumulation was 2.7% for ISO-stimulated cells and 1.4% for the ISO + OXO-stimulated cells. Basal accumulation was constant at all times examined and corresponded to A/A_o values of 0.05 and 0.09 for ISO and ISO + OXO, respectively.

Bottom panel: Plot of the natural logarithm of A/A_o versus time. In five experiments carried out in triplicate, a single regression line accounted for an average of 92% of the variability (r = 0.96) in the data from cells incubated with ISO. A single regression line could account for only 60% of the variability (r = 0.78) during degradation of cyclic AMP in the presence of ISO plus OXO.

Consideration of the data for degradation in the presence of OXO, based on a two-component analysis with the fast component expressed from 0 to 1 min and the slower component from 1.5 to 5 min, accounted for 84% and 81% of the variability within the respective time intervals (r = 0.92 and 0.89, respectively). Average k_{deg} values for the fast component and slow component in the presence of OXO were 1.08 ± 0.05 min^{-1} and 0.25 ± 0.06 min^{-1} (n = 5 experiments).

absence of added Ca^{2+}, the inhibitory effects of OXO were markedly reduced (Fig. 6). The level of inhibition sharply increased when Ca^{2+} concentrations in the medium were restored to 0.5–1 mM. Maximal inhibition was observed between 2 and 5 mM Ca^{2+}. Experiments designed either to chelate the Ca^{2+} in the normal DMEM-Hepes incubation medium using EGTA or to block the entry of Ca^{2+} into the cell by the addition of Co^{2+} yielded similar results; that is, the reduction of the accumulation of [^{3}H]cyclic AMP in the presence of OXO was reversed as the concentration of EGTA or Co^{2+} increased (data not shown). In the presence of 20 mM Co^{2+}, OXO had no

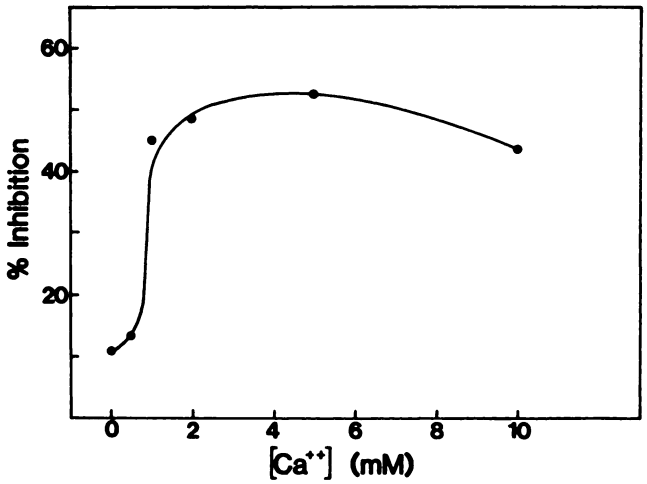

FIG. 6. *Calcium dependence of OXO-mediated inhibition of [^{3}H] cyclic AMP accumulation*

1321N1 cells were prelabeled with [^{3}H]adenine and then were washed in DMEM-Hepes + 10 mM EGTA followed by two washes with Hepes-buffered saline (pH 7.4). Incubations were carried out with the indicated Ca^{2+} concentrations and ISO (10 μM) or ISO (10 μM) + OXO (100 μM) for a period of 5 min. The data are plotted as the inhibition of [^{3}H]cyclic AMP accumulation by OXO at the indicated concentrations of added Ca^{2+}. Each point represents the mean of four determinations.

effect on cyclic AMP levels. When cyclic AMP degradation was measured in the absence of Ca^{2+}, no difference in k_{deg} values was observed between cultures incubated in the absence versus in the presence of OXO (Fig. 7).

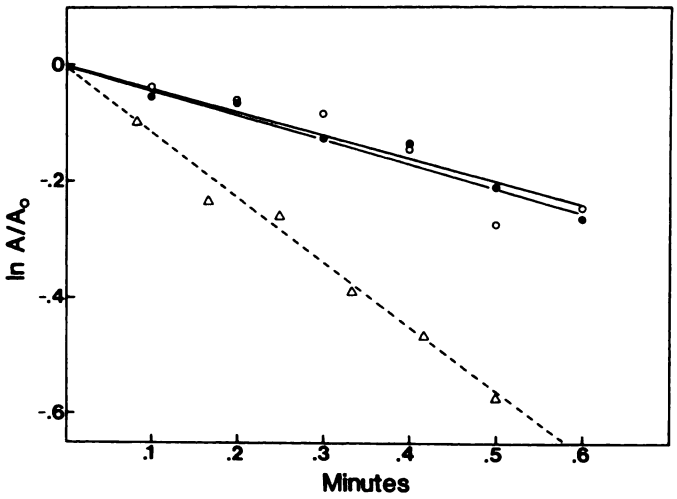

FIG. 7. *Effect of OXO on cyclic AMP degradation in the absence of Ca^{2+}*

Cells were prelabeled with [^{3}H]adenine and washed once with Hepes-buffered saline + 5 mM EGTA and once with Hepes-buffered saline. Cells were incubated with 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○) for 5 min prior to stopping the synthetic reaction with propranolol (10 μM final concentration). At the indicated times the cells were then fixed by aspiration of the medium and addition of 1.0 ml of 5% trichloroacetic acid. Total [^{3}H]cyclic AMP accumulation at the time of propranolol addition was 1.41% for the ISO-stimulated cells and 1.25% for cells stimulated with ISO plus OXO. Rate constants for cyclic AMP degradation were 0.46 min^{-1} and 0.42 min^{-1} in the absence and presence of OXO, respectively. Cyclic AMP degradation in the presence of OXO in normal Ca^{2+}-containing medium was also measured (△). The k_{deg} under this condition was 1.08 min^{-1}. The results are representative of three experiments.

TABLE 2

Inhibition of cyclic AMP accumulation in 1321N1 cells by the ionophore A23187

The data are presented as the percentage conversion of [^{3}H]ATP to [^{3}H]cyclic AMP, determined as described under Methods. Cyclic AMP levels were measured 5 min after addition of drugs at the following concentrations: ISO, 10 μM; OXO, 100 μM; A23187, 0.1 μM; IBMX, 0.5 mM; atropine, 1 μM. The data are the means ± standard error of the mean of four determinations and are representative of results from three similar experiments.

Additions	% Conversion of [^{3}H]ATP to [^{3}H]cyclic AMP	% Inhibition
ISO	2.49 ± 0.11	
ISO + A23187	1.07 ± 0.07	57.0
ISO + OXO	1.07 ± 0.05	57.0
ISO + OXO + A23187	1.22 ± 0.02	51.0
ISO	2.30 ± 0.10	
ISO + A23187	0.532 ± 0.03	76.9
ISO + IBMX	3.15 ± 0.10	—
ISO + IBMX + A23187	4.14 ± 0.11	—
ISO + A23187 + atropine	0.987 ± 0.06	57.1

In contrast to the results obtained in experiments designed to eliminate Ca^{2+} from the extracellular medium, the divalent cation ionophore, A23187, mimicked the effects of muscarinic receptor stimulation. As summarized in Table 2, the presence of A23187 greatly reduced the accumulation of cyclic AMP in response to stimulation by ISO. A 57–77% inhibition of accumulation was observed with A23187 as compared with 57% inhibition by OXO under similar conditions. Co-addition of OXO and A23187 at maximally effective concentrations caused no further reductions of cyclic AMP levels than was observed with either agent alone (Table 2). The effects of 0.1 μM A23187 were completely reversed by 0.5 mM IBMX, but not by 1 μM atropine, suggesting that A23187 activates phosphodiesterase by a mechanism that does not involve the muscarinic receptor. Although experiments have not been carried out to test directly the effects of A23187 on Ca^{2+} fluxes, the reported effects of this drug (29), together with the present data, suggest that alteration in intracellular Ca^{2+} alone appears to be a condition sufficient for activation of phosphodiesterase in 1321N1 cells. This possibility is also supported by the observation of an enhanced rate of cyclic AMP degradation in the presence of A23187 (Fig. 8). Following a lag of approximately 30 sec, cyclic AMP degradation occurred at an enhanced rate (k_{deg} = 1.11 min^{-1}) as compared with degradation in control cells (k_{deg} = 0.52 min^{-1}). Addition of carbachol in the same experiment yielded a k_{deg} value of 1.08 min^{-1} (data not shown).

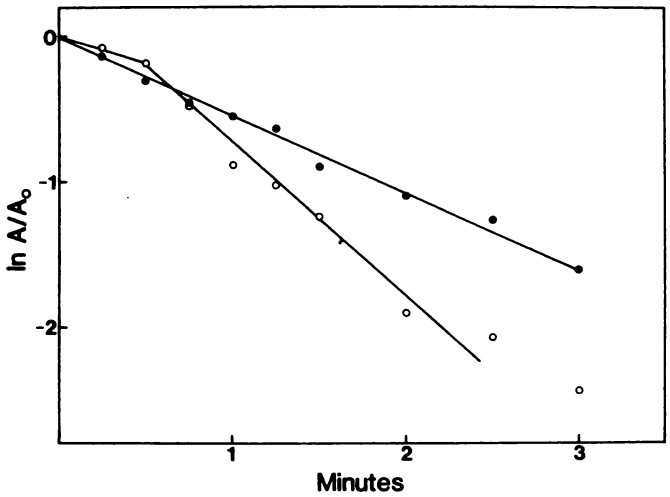

FIG. 8. *Degradation of cyclic AMP in the presence of A23187*

After stimulation of whole cells with 10 μM ISO for 5 min, a 50-μl aliquot of propranolol (●) or propranolol plus A23187 (○) was added to the medium to give final concentrations of 10 μM and 1 μM, respectively. Cyclic AMP levels were then determined as a function of time. The k_{deg} values were 0.51 min^{-1} for control cells and, after a lag of approximately 0.5 min, 1.11 min^{-1} in the presence of A23187. Each point was determined in quadruplicate. Standard errors were less than 12% of the mean.

Interaction of muscarinic receptors with adenylate cyclase. A 2- to 3-fold increase in the rate of degradation of cyclic AMP in the presence of OXO can account for the 50–60% decrease in cyclic AMP levels in intact 1321N1 cells. However, in light of the inhibition of adenylate cyclase activity by muscarinic receptor agonists observed in membrane preparations from other tissues, it was of interest to examine the efficacy of OXO for inhibition of adenylate cyclase activity in cell-free preparations from 1321N1 cells. As is illustrated in Table 3, a significant inhibition of adenylate cyclase by OXO was not observed with 1321N1 cell membranes. These data illustrate a typical result from a series of experiments over a 20-month period, during which time various approaches were used in an attempt to observe inhibition of adenylate cyclase activity in 1321N1 cells. In comparison, OXO inhibited enzyme activity by approximately 35% in heart membranes under the same conditions (Table 3) and by 20–40% in membranes from WI-38 fibroblasts and NG 108–15 neuroblastoma × glioma cells (data not shown).

Phosphodiesterase activity in cell-free preparations. A series of experiments was also carried out to determine whether a muscarinic receptor-mediated activation of phosphodiesterase activity could be observed in cell-free

TABLE 3

Effect of muscarinic receptor agonists on adenylate cyclase activity in cell-free preparations from 1321N1 astrocytoma cells and rat heart

Adenylate cyclase was measured as described under Methods in the presence of the indicated drugs. OXO, carbachol, and ISO were present at concentrations of 100, 100, and 10 μM, respectively. The data are the means ± standard error of the means for the indicated number of experiments.

Additions	Adenylate cyclase activity	
	1321N1 Cells	Rat heart
	pmoles/min/mg	
Basal	12.3 ± 0.4 (n = 4)	20.0 ± 2.0 (n = 6)
OXO	12.3 ± 0.1	13.0 ± 1.5
Carbachol	12.8 ± 0.3	—
ISO	74.1 ± 2.4 (n = 4)	53.7 ± 8.2 (n = 6)
ISO + OXO	72.7 ± 2.0	35.4 ± 4.9
ISO + carbachol	72.9 ± 0.9	—

preparations. Lineweaver-Burk analysis of phosphodiesterase activity in homogenates of 1321N1 cells suggested the existence of two forms of the enzyme. The low-affinity form could be measured at substrate concentrations of 20–400 μM, and the high-affinity form could be measured at substrate concentrations of 0.1–10 μM. Cells were treated with 100 μM OXO for 5 min, the cells were rapidly lysed, and kinetic analyses of phosphodiesterase activity were carried out with a cell-free preparation. Neither the K_m for the high-affinity form (control = 3.7 ± 1.3 μM; OXO-treated = 4.4 ± 1.4 μM; n = 4) nor that of the low-affinity form (control = 130 μM; OXO-treated = 152 μM; n = 2) of the enzyme was changed by preincubation of 1321N1 cells with OXO (Fig. 9A). The V_{max} of the high-affinity form varied greatly (127–1375 pmoles/min/mg of protein) from experiment to experiment. In each experiment a small increase (36.2 ± 9.2% over control; n = 4) in activity occurred in OXO-pretreated cells. The V_{max} for the low-affinity form of phosphodiesterase was 5522 pmoles/min/mg of protein (n = 2) for control homogenates and 6188 pmoles/min/mg of protein (n = 2) after OXO pretreatment of cells. The addition of 100 μM OXO to homogenates had no effect on phosphodiesterase activity (data not shown). Phosphodiesterase activity in soluble and particulate fractions from OXO (100 μM)-pretreated 1321N1 cells was also examined in time course experiments (Fig. 9B) and with a Lineweaver-Burk analysis (data not shown). OXO pretreatment had no effect on activity in either fraction.

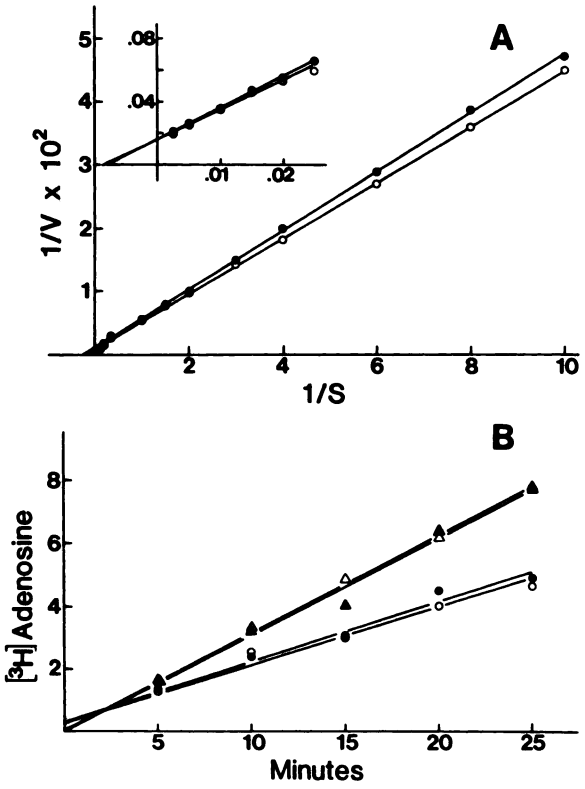

FIG. 9. *Phosphodiesterase activity in cell-free preparations*

A. Lineweaver-Burk analysis of cyclic AMP phosphodiesterase activity in homogenates prepared from 1321N1 cells. Cells were treated with 100 μM OXO (○) or vehicle (●) for 5 min. Homogenates were then prepared and phosphodiesterase activity measured as described under Methods. Data are plotted as the reciprocal of substrate concentration (× 10^{-6} M) on the abscissa versus the reciprocal of velocity (minutes × milligram per picomole) on the ordinate. The data are representative of results from five experiments. *Inset*, The plot of the data obtained at high substrate concentrations (40–400 μM).

B. Phosphodiesterase activity in soluble and particulate fractions from 1321N1 cells. Cells were treated with vehicle (●, ▲) or 100 μM OXO (○, △) for 5 min and then soluble and particulate fractions were prepared as described under Methods. Phosphodiesterase activity in the soluble (▲, △; picomoles of [^{3}H]adenosine formed per milligram of protein × 10^{-4}) and particulate (●, ○; picomoles of [^{3}H]adenosine formed per milligram of protein × 10^{-3}) fractions was measured at a substrate concentration of 100 μM. The results are representative of four experiments.

DISCUSSION

On the basis of experiments with [^{3}H]QNB, the binding properties of muscarinic cholinergic receptors of 1321N1 cells appear to be very similar to those described for muscarinic cholinergic receptors in a variety of tissues (30–33). Antagonists and agonists of muscarinic receptors competed for [^{3}H]QNB binding sites with affinities similar to those reported by others (30–33), and the K_D of [^{3}H]QNB as determined by either kinetic or equilibrium binding experiments was similar to that previously reported (31). The rate constant for dissociation of [^{3}H]QNB determined in 1321N1 cell membranes was several-fold lower than that observed in a number of other tissues. However, it should be pointed out that many studies have been carried out with racemic [^{3}H]QNB under conditions in which both stereoisomers may be binding to muscarinic receptors. Thus, the more rapid and often more complex dissociation of [^{3}H]QNB observed in other studies (32, 33) may reflect a more rapid dissociation of the less active (+)-stereoisomer followed by a slower-occurring dissociation of the (−)-stereoisomer (34).

The failure to observe substantial inhibition of adenylate cyclase activity by OXO in membrane preparations from 1321N1 cells was surprising initially in light of the marked reduction in cyclic AMP accumulation observed in intact cells in the presence of OXO (Table 1; ref. 19). In a variety of other systems in which muscarinic receptor agonists reduce cyclic AMP levels in intact cells, agonist-induced reductions in adenylate cyclase activities can be observed in membrane preparations. In these systems, guanine nucleotides cause a marked reduction in agonist affinities as determined in radioligand-binding experiments (35–37). Such an effect is thought to be reflective of fundamental events involved in the coupling of receptors to a guanine nucleotide regulatory component of adenylate cyclase. Although guanine nucleotides markedly reduce the affinity of agonists binding to *beta*-adrenergic receptors of washed 1321N1 cell membranes (18), no effect of nucleotides on the interaction of agonists with muscarinic receptors was observed.[3] Our data do not completely rule out the possibilities that coupling between the muscarinic receptor and components of the adenylate cyclase system is lost during preparation of membranes from 1321N1 cells or that assay conditions for observing inhibition of adenylate cyclase are not optimal. However, under similar conditions we consist-

[3] R. B. Meeker and T. K. Harden, unpublished observations.

ently observe a 20–40% inhibition of adenylate cyclase activity with membrane preparations from WI-38 fibroblasts, NG 108-15 neuroblastoma × glioma cells, or rat heart.

Kinetic studies of cyclic AMP accumulation in 1321N1 cells support the idea that coupling of muscarinic receptors to the synthetic component of the cyclic AMP system is relatively unimportant in determining the effect of muscarinic agonists on cyclic AMP levels. Indeed, three different results indicate that the primary action of muscarinic receptor stimulation involves an activation of phosphodiesterase. First, in the presence of OXO, the time to maximal cyclic AMP accumulation is reduced by approximately 3-fold. Since the time to maximal levels should be inversely related to the rate of cyclic AMP degradation (38), these data are more consistent with an increase in the rate of cyclic AMP degradation than with an OXO-mediated inhibition of adenylate cyclase. Second, the presence of IBMX abolished both the inhibitory effect of OXO on cyclic AMP accumulation and the difference in the time to maximal cyclic AMP accumulation determined in the absence versus the presence of OXO. Thus, reduction by IBMX of the contribution of phosphodiesterase to cyclic AMP accumulation eliminates the influence of muscarinic receptors on cyclic AMP accumulation. Finally, direct measurement of the rate of cyclic AMP degradation after blockade of synthesis indicates that muscarinic receptor activation results in a 2.5- to 3-fold increase over control in the rate of cyclic AMP degradation. These data provide the most conclusive evidence for a muscarinic receptor-mediated activation of phosphodiesterase, since adenylate cyclase activity is blocked by the presence of propranolol in these experiments, and therefore, synthesis of nucleotide does not contribute significantly to cyclic AMP levels. The kinetic analysis also suggests that at least two pools or forms of phosphodiesterase may be involved in cyclic AMP metabolism in 1321N1 cells with only one of these components subject to regulation by muscarinic receptors. That is, degradation of cyclic AMP in the presence of OXO occurred by a rapid component accounting for at least 80% of the cyclic AMP that was degraded and a slower component with a rate of degradation similar to that observed in the absence of muscarinic receptor activation. Alternatively, rapid desensitization of the muscarinic receptor system might account for the apparent biphasic nature of cyclic AMP degradation in the presence of OXO (20). Accurate analysis of this component is difficult, since cyclic AMP levels approach basal levels during the time when the slower component can be observed.

As was previously shown by Gross and Clark (19), the capacity of muscarinic receptor agonists to reduce cyclic AMP accumulation in 1321N1 cells is markedly inhibited in cells maintained in the absence of free Ca^{2+} (Fig. 6). Such a finding is again consistent with an interaction between muscarinic receptors and degradative activity in 1321N1 cells, since Ca^{2+} has been shown in a variety of systems to be involved in the activation of phosphodiesterase (39). Thus, elevation of cytoplasmic levels of Ca^{2+} is likely to be a necessary step for muscarinic receptor-mediated reduction of cyclic AMP levels in 1321N1 cells. The data indicating that in the absence of Ca^{2+} the k_{deg} for cyclic AMP in the presence of OXO is the same as the k_{deg} for control cells suggest that muscarinic receptors principally alter cyclic AMP levels through Ca^{2+}-dependent changes in phosphodiesterase activity. Results from experiments with A23187 add further support to the idea that phosphodiesterase activity in these cells is regulated by Ca^{2+}; lack of additivity of the inhibitory effects of A23187 and OXO are at least consistent with these two drugs reducing cyclic AMP levels through a common mechanism.

Direct analysis of cyclic AMP phosphodiesterase activity in broken-cell preparations provided inconclusive data. The small increases in V_{max} that were observed in preparations from OXO-treated cells could not account quantitatively for the 2.5- to 3-fold increase in degradative activity measured in the presence of OXO in intact cells. However, since activation of cyclic AMP phosphodiesterase appears to be linked to Ca^{2+} mobilization or entry into 1321N1 cells, it is not surprising that muscarinic receptor-mediated regulation of enzyme activity is lost after cell lysis.

As previously stated, reduction in intracellular cyclic AMP levels in a variety of tissues has been thought to occur principally through an inhibitory coupling of muscarinic receptors to adenylate cyclase. However, data from the present study indicate that this need not be the case, and indeed, activation of phosphodiesterase subsequent to stimulation of muscarinic receptors appears to account for the effects of OXO on cyclic AMP levels in 1321N1 cells. At least two previous studies suggest that this mechanism may not be restricted to the muscarinic receptor system of 1321N1 astrocytoma cells. Although inhibition of cyclic AMP synthesis appears to be the principal mechanism of cholinergic agonist-induced lowering of cyclic AMP levels in cultured WI-38 fibroblasts (40),[4] there is also evidence that in the presence of a muscarinic receptor agonist, there is increased turnover of cyclic AMP (14). Moreover, Van Sande *et al.* (13) concluded that the principal mechanism through which muscarinic cholinergic receptors reduced cyclic AMP accumulation in dog thyroid slices was through activation of phosphodiesterase. Thus, it may prove to be an oversimplification to think of muscarinic and other (e.g., *alpha*$_2$-adrenergic, opiate, adenosine) receptors as lowering cyclic AMP levels through effects on nucleotide synthesis alone. Indeed, more complete examination of the kinetics of cyclic AMP accumulation in cells in which receptors are thought to be linked in an inhibitory manner to the cyclic AMP-generating system may reveal that hormonal effects on phosphodiesterase activity are a relatively common occurrence. 1321N1 Astrocytoma cells, which express relatively high concentrations of muscarinic receptors and marked effects of agonists of these receptors on phosphodiesterase activity, should prove to be a useful system for the further elucidation of the mechanism involved in this novel means of hormonal regulation of the cyclic AMP system.

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[4] M. M. Smith and T. K. Harden, unpublished observations.

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