Biochemical Pharmacology, Vol. 52, pp. 643–658, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00339-5

Stimulation of Cyclic AMP Accumulation and Phosphoinositide Hydrolysis by M_3 Muscarinic Receptors in the Rat Peripheral Lung

Evette E. Esqueda, Edward H. Gerstin, Jr.,**
Michael T. Griffin† and Frederick J. Ehlert‡*

*Department of Pharmacology, College of Medicine, University of California, Irvine, CA 92717; and †Department of Chemistry, Chapman University, Orange, CA 92667, U.S.A.

ABSTRACT. The effects of oxotremorine-M (oxo-M), a muscarinic agonist, on cyclic AMP (cAMP) accumulation in slices of the rat peripheral lung were investigated. Oxo-M stimulated cAMP accumulation in a concentration-dependent manner with an EC_{50} value of 4.2 μM and a maximal effect of 2.4 ± 0.39-fold over basal. In the presence of forskolin (25 μM), the maximal effect of oxo-M was increased to 14.1 ± 4.0-fold over basal. Forskolin alone caused a 5.9 ± 2.2-fold increase in cAMP relative to basal; therefore, the combination of both drugs was more than additive. The effects of oxo-M on cAMP accumulation were unaffected by tetrodotoxin, indicating that the action of oxo-M was not mediated by neuronal release of neurotransmitters. Oxo-M had a small inhibitory effect on cAMP in a homogenate preparation, indicating that the stimulatory response to oxo-M in slices of the lung is not due to direct stimulation of adenylyl cyclase. Characterization of the oxo-M potentiation of forskolin-stimulated cAMP accumulation using different muscarinic antagonists yielded calculated pK_B values that agreed with binding affinities for the M_3 subtype. Oxo-M elicited phosphoinositide hydrolysis in the lung, and the nature of the antagonism of this response was also consistent with that expected for an M_3-mediated response. cAMP accumulation in the presence of oxo-M (100 μM), forskolin (12 μM), or both drugs combined was inhibited by indomethacin (1 μM). These results demonstrate that the M_3 receptor stimulates cAMP accumulation and phosphoinositide hydrolysis in the rat peripheral lung, and the mechanism for cAMP stimulation may involve arachidonic acid metabolites. BIOCHEM PHARMACOL 52;4:643–658, 1996.

KEY WORDS. lung, muscarinic receptors; lung, cAMP, muscarinic stimulation; lung, phosphoinositide hydrolysis, muscarinic stimulation; muscarinic receptor, stimulation of cAMP; muscarinic receptor, rat peripheral lung; muscarinic receptor, phosphoinositide hydrolysis

The muscarinic receptors can be divided into five subtypes (M_1–M_5) on the basis of their primary sequence and pharmacological properties [1]. Although there is considerable structural homology in the transmembrane spanning regions of the subtypes, there is divergence in the third cytoplasmic loop that confers selectivity for different G proteins and their signaling pathways [2]. The M_1, M_3, and M_5 subtypes couple to phosphoinositide hydrolysis, whereas the M_2 and M_4 subtypes preferentially couple to inhibition of adenylyl cyclase [3]. However, in transfected cells expressing high densities of M_2 and M_4 receptors, a weak, pertussis toxin-sensitive phosphoinositide response has been observed. In contrast, the phosphoinositide response elicited by the M_1, M_3, and M_5 subtypes is usually pertussis toxin insensitive [4, 5]. Direct stimulation of adenylyl cyclase also has been observed. Onali *et al.* [6] have demonstrated in homogenates of the rat olfactory bulb that the M_4 receptor stimulates adenylyl cyclase through a pertussis toxin-sensitive G protein [7]. Also, when transfected into CHO[§] cells, the M_1 receptor has been shown to stimulate adenylyl cyclase in cellular homogenates [8, 9]. In the intact cell, more complicated signaling mechanisms exist due to the accumulation of intracellular mediators such as Ca^{2+}. For example, M_1 and M_3 muscarinic receptors stimulate cAMP accumulation in intact AL9 cells and bovine iris, respectively, and this effect appears to be dependent upon phosphoinositide hydrolysis, which causes a mobilization of Ca^{2+} and an activation of Ca^{2+}–calmodulin-dependent adenylyl cyclase [10–12]. Similar observations have been made in intact SH-SY5Y cells [13]. However, Baumgold *et al.* [14] reported that in SK-N-SH human neuroblastoma

‡ Corresponding author: Dr. Frederick J. Ehlert, Department of Pharmacology, University of California, Irvine, College of Medicine, Irvine, CA 92717. Tel. (714) 824-6208; FAX (714) 824-4855.
Received 26 August 1995; accepted 12 March 1996.

§ *Abbreviations:* AF-DX 116, [[2-[(diethylamino)methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one; AMP, cyclic AMP; CHO, Chinese hamster ovary; HHSiD, hexahydrosiladifenidol; IBMX, isobutylmethylxanthine; IP_3, inositol-1,4,5-trisphosphate; KRB, Krebs–Ringer bicarbonate; NMS, *N*-methylscopolamine; oxo-M, oxotremorine-M; PGE_2, prostaglandin E_2; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; and TCA, trichloroacetic acid.

cells, a muscarinic receptor is able to stimulate cAMP accumulation independent of elevated intracellular calcium levels. Other investigators have proposed that muscarinic-stimulated cAMP accumulation may be mediated by mechanisms involving cGMP or PKC as well as calcium [15–18].

Muscarinic receptors in the lung have been identified in several species and have been shown to be involved in airway constriction, mucus secretion, and prejunctional control of neurotransmitter release. The subtypes present in the central airways are most commonly M_2 and M_3 [19]. Functional studies in the trachea have shown M_2-mediated inhibition of adenylyl cyclase and M_3-mediated phosphoinositide hydrolysis resulting in intracellular calcium release and contraction [20–24]. Species differences arise with regard to the distribution of muscarinic receptor subtypes in the peripheral lung. The peripheral lung of rats contains predominantly M_2 receptors [25, 26], whereas those of the human [27, 28] and rabbit [29, 30] contain predominantly M_1 and M_4 receptors, respectively. The functional significance of these receptors is unclear since the peripheral lung consists of many different cell types; however, it is possible that the localization of these receptors may include smooth muscles, mucus glands, ganglia, or pulmonary vessels [31]. Further studies characterizing these receptors and their function could be useful in developing therapies for obstructive airway diseases.

In this study, we have investigated the effects of a muscarinic receptor on cAMP accumulation in the rat peripheral lung. We have found that oxo-M, a muscarinic agonist, potentiates forskolin-stimulated cAMP production and stimulates phosphoinositide hydrolysis. Both of these responses are mediated by the M_3 subtype, although the majority of muscarinic receptors present are of the M_2 subtype.

MATERIALS AND METHODS

Materials

All of the drugs used were obtained from the Sigma Chemical Co., St. Louis, MO, with the exception of the following: pirenzepine, methoctramine, and oxotremorine-M, Research Biochemicals Inc., Natick, MA; AF-DX 116, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT; HHSiD, Dr. Günter Lambrecht, University of Frankfurt, Frankfurt, Germany; [α-^{32}P]ATP, ICN Radiochemicals, Irvine, CA; and [^{3}H]adenine, [^{3}H]inositol, and [^{3}H]NMS, Dupont-New England Nuclear, Boston, MA.

cAMP Accumulation

cAMP was measured using a modification of the [^{3}H]adenine-prelabeling method described by Daly *et al.* [32]. Male Sprague–Dawley rats (150–250 g) were killed by decapitation. Their lungs were removed and placed into ice-cold KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose) gassed with O_2/CO_2 (19:1). Peripheral sections of the lung were dissected away carefully from the majority of the tissue by cutting along the outer edges and the surfaces of each lobe. The tissue was cross-chopped (350 × 350 μm) with a McIlwain tissue chopper, and the resulting slices were transferred to an Erlenmyer flask with KRB buffer. The suspension of slices was degassed by reduced pressure which caused the slices to settle. The slices were immediately washed three times with fresh KRB buffer gassed with O_2/CO_2 (19:1). The tissue was incubated with [^{3}H]adenine (50 μCi) for 40 min at 37° in 10 mL of KRB buffer. Aliquots of gently packed tissue were added to tubes containing KRB buffer (0.7 mL), 0.5 mM IBMX, and various drugs as described under Results. The tissue was incubated for 10 min at 37°. All measurements were done in triplicate. During antagonism experiments, slices were incubated with the appropriate antagonist for 30 min prior to the 10-min incubation with agonist. Reactions were stopped by the addition of 0.3 mL TCA (30%, w/v) and homogenization with a polytron. [^{3}H]cAMP was measured in the TCA extracts as described previously [33].

In some experiments, a mass assay was run simultaneously with the [^{3}H]adenine-prelabeling technique. Triplicate TCA extracts from above were combined and subsequently divided into two aliquots: one for estimation of cAMP as described above and the other for mass determination. The TCA extract (1 mL) for the mass assay was applied to a cation exchange column (3.0 mL of Dowex AG 50W-X4, 200–400 mesh) and washed three times with water (1 mL), and the eluates were discarded. The cAMP was eluted with 7 mL of water, and the mass of cAMP present in the eluate was determined using the Rainen cAMP [^{125}I] Radioimmunoassay Kit (Dupont, Boston, MA). Protein was estimated using the method of Lowry *et al.* [34].

Phosphoinositide Hydrolysis

Oxo-M-stimulated phosphoinositide hydrolysis was measured as described previously [33] using a modification of the method described by Berridge *et al.* [35]. Slices of peripheral lung were prepared as described above, washed three times with KRB buffer, and allowed to equilibrate at 37° for 30 min. The slices were incubated for 90 min in a final volume of 4 mL of KRB buffer containing 40–50 μCi of [^{3}H]*myo*-inositol, gassed continually with O_2/CO_2 (19:1). Aliquots of gently packed tissue were added to tubes containing KRB buffer (0.35 mL), LiCl (10 mM), and various drugs as described under Results. The tissue was incubated for 30 min at 37°. During antagonism experiments, slices were incubated with the appropriate antagonist for 30 min prior to the 30-min incubation with agonist. The reactions were stopped as previously described [33]. The amount of [^{3}H]inositol phosphate formed (cpm) is expressed as a percentage of the total amount of radioactivity in the organic phase plus the inositol phosphate fraction, to correct for minor variations in the amount of tissue added to each assay.

Adenylyl Cyclase Activity

Lung tissue was obtained as described above, weighed, and suspended in ice-cold 30 mM sodium HEPES buffer, pH

7.5, containing 3 mM dithiothreitol and 1 mM EGTA. The tissue was homogenized with a Polytron homogenizer, yielding a final tissue concentration of 50 mg tissue (original wet weight) per mL of buffer. An aliquot (0.05 mL) of this homogenate was incubated in a final volume of 0.2 mL containing 30 mM sodium HEPES, pH 7.5, 0.25 mM ATP, [α-^{32}P]ATP (1 μCi), 1 mM cAMP, 0.75 mM dithiothreitol, 0.63 mM EGTA, 0.1 mM GTP, 0.5 mM IBMX, 10 mM KCl, 5 mM $MgCl_2$, 5 mM phosphocreatine, 30 U/mL creatine phosphokinase, 0.05% bovine serum albumin, and various drugs as described under Results. The incubation lasted for 10 min at 37°. The reaction was stopped by the addition of an aliquot (0.1 mL) containing 40 mM ATP, 1.4 mM cAMP, and 0.1 mM sodium dodecyl sulfate, titrated to pH 7.5 with Tris base. An aliquot (0.8 mL) of water containing [^{3}H]cAMP (approximately 4000 cpm) was added as an internal standard, and [^{32}P]cAMP was determined using the method of Salomon *et al.* [36].

Radioligand Binding

The competitive inhibition of [^{3}H]NMS binding to the lung and to CHO cells transfected with muscarinic receptor subtypes was measured as described previously [37]. The lung tissue was dissected from rats as described above and homogenized with a Polytron homogenizer at setting number five in 20 mL of ice-cold HEPES buffer (30 mM sodium HEPES, 100 mM NaCl, 1 mM $MgSO_4$, 0.5 mM EGTA, pH 7.4). The homogenate was centrifuged at 31,000 *g* for 10 min. The resulting supernatant was discarded, and the pellet was resuspended in HEPES buffer to 18.75 mg/mL (based on original wet weight). An aliquot (0.8 mL) of tissue homogenate was incubated in a final volume of 1 mL for 30 min at 25° with [^{3}H]NMS (0.5 nM) and various concentrations of pirenzepine as described under Results. For experiments on CHO cells, an aliquot (0.8 mL) of cell suspension (see below) was incubated in a final volume of 1 mL of modified KRB buffer (124 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 26 mM $NaHCO_3$ and 10 mM sodium HEPES, pH 7.4) with [^{3}H]NMS and various drugs for 1 hr at 30°. Non-specific binding was defined as the residual binding in the presence of 1.0 μM atropine. All assays were run in triplicate.

Cell Culture

CHO cells stably expressing the human M_1–M_5 subtypes of the muscarinic receptor were provided by Dr. Mark Brann (University of Vermont, Burlington, VT). These cells were grown in Dulbecco's Modified Eagle's Medium with high glucose plus L-glutamine, supplemented with 7% fetal bovine serum, sodium bicarbonate (3.7 g/L), and in the presence of 500 U penicillin and 500 U streptomycin. Cells were grown and maintained in a 37° incubator with a humidified atmosphere containing 5% CO_2. Cells were detached by incubation at 37° for 5 min with 2 mL of a solution of trypsin–EDTA in normal saline. Tissue culture flasks (75 cm^2) inoculated with 2.5×10^5 cells were typically confluent in 3 days. Cells were detached as described above and centrifuged. The resultant pellets were stored at −70° until used in the binding assay. On the day of the binding assay, cell pellets were thawed and resuspended in modified KRB buffer.

Calculations

The EC_{50} values (concentration of drug eliciting a half-maximal response) and maximal responses of oxo-M for stimulating phosphoinositide hydrolysis and cAMP accumulation were estimated by nonlinear regression analysis of the concentration–response curves according to a four-parameter logistic equation as described previously [38].

The high (K_H) and low (K_L) affinity dissociation constants of pirenzepine were estimated by nonlinear regression analysis of the pirenzepine/[^{3}H]NMS competition curve according to a two-site binding equation as described previously [37]. The apparent dissociation constants were corrected for the competitive effect of [^{3}H]NMS assuming a dissociation constant (K_D) of [^{3}H]NMS of 0.5 nM. This value was estimated previously at M_2 receptors in the rat heart [39].

Statistics

An analysis of variance technique was used to determine whether there were significant differences between the K_B values of antagonists measured by antagonism of the cAMP response and those measured by antagonism of the phosphoinositide response. Similarly, this technique was also used to compare K_B values from the functional studies with the corresponding binding affinities (K_D values) measured at the M_1–M_5 subtypes of the muscarinic receptor. In this analysis, all dissociation constants (i.e. K_B and K_D values) were converted to logarithms. For each antagonist, the following ratio (R) was calculated:

$$\text{Log } R_1 = \text{Log}\left(\frac{K'_{B1}}{K''_{B1}}\right)$$

in which K'_{B1} denotes the dissociation constant of the first antagonist estimated in one type of assay (e.g. cAMP assay) and K''_{B1} denotes that determined in another type of assay (e.g. phosphoinositide assay or binding assay). Four ratios were determined (R_1–R_4) corresponding to the four antagonists used in this study (i.e. AF-DX 116, HHSiD, methoctramine, and pirenzepine). The variance of each Log ratio (S_R^2) was estimated as the sum of the variances of the Log K'_B ($S_{K'}^2$) and Log K''_B ($S_{K''}^2$) values (i.e. $S_R^2 = S_{K'}^2 + S_{K''}^2$). If the K'_B and K''_B values are the same, then their corresponding ratios (R_1–R_4) should all be the same and equivalent to one [i.e. Log(R) = 0]. Consequently, one-way analysis of variance was used to test these two criteria.

RESULTS

cAMP Accumulation

The effects of oxo-M and forskolin on cAMP accumulation in slices of the rat peripheral lung are shown in Fig. 1.

When measured using the mass assay described under Materials and Methods (see Fig. 1A), basal levels of cAMP were 3.2 ± 0.15 nmol/mg protein. Oxo-M (100 μM) had a small but nonsignificant effect on the mass of cAMP (3.4 ± 0.31 nmol/mg protein), whereas forskolin (12 μM) caused a 2.2-fold increase (7.2 ± 0.41 nmol/mg protein) relative to basal. In the presence of both oxo-M (100 μM) and forskolin (12 μM), the mass of cAMP increased 2.8-fold (9.1 ± 0.60 nmol/mg protein) relative to basal. The effects of oxo-M and forskolin on cAMP accumulation in the lung were also investigated using the [^{3}H]adenine-prelabeling technique described under Materials and Methods (see Fig. 1B). These measurements were made on the same samples from which mass was estimated in Fig. 1A. Using this technique, the basal level of cAMP was estimated at 1.2 ± 0.09% (i.e. expressed as a percentage of the total [^{3}H]adenine-labeled nucleotides). Oxo-M (100 μM) caused a 1.6-fold increase in cAMP accumulation (1.8 ± 0.13%) relative

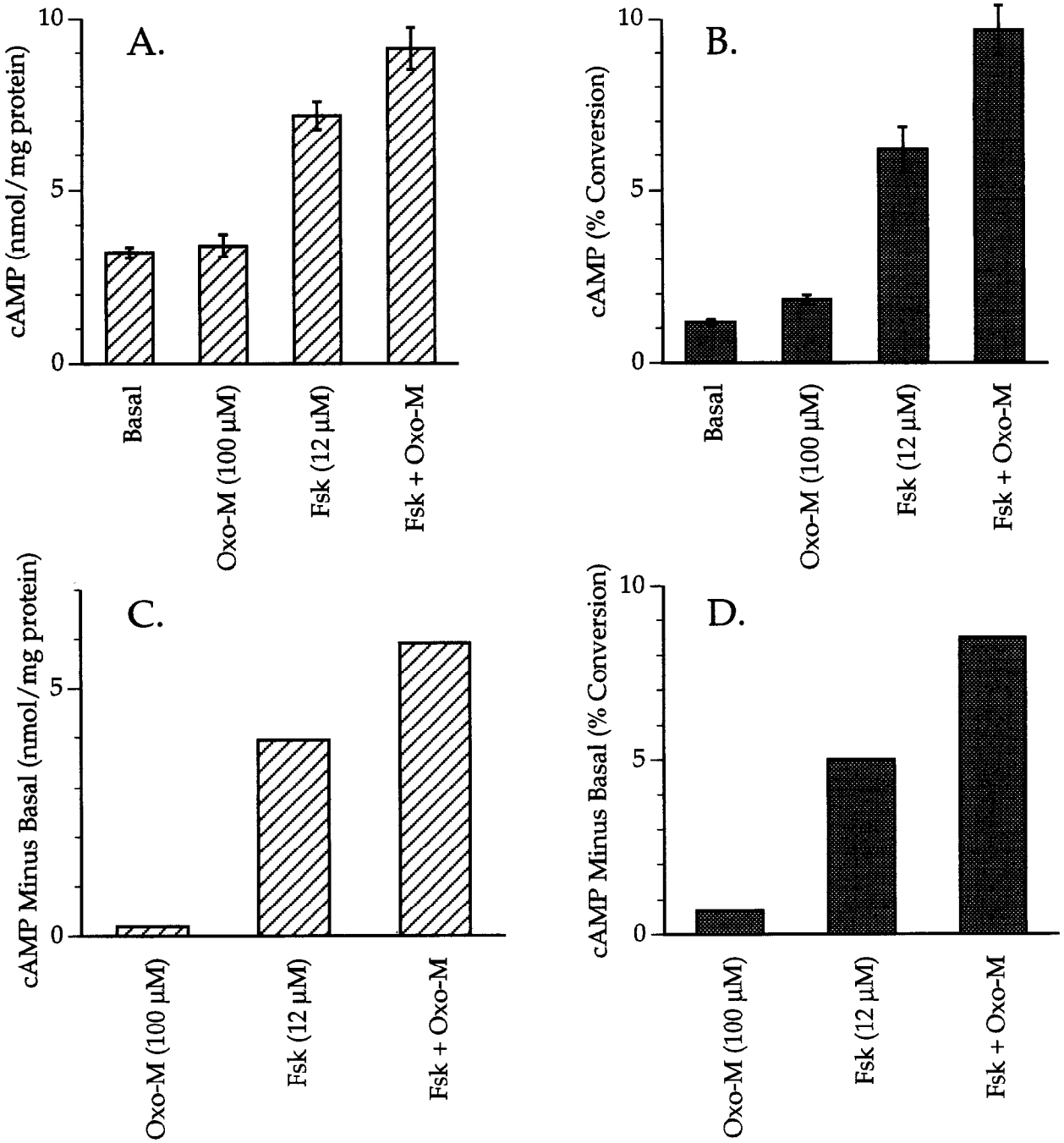

FIG. 1. Effects of oxo-M, forskolin (Fsk), and their combination on cAMP accumulation in rat peripheral lung slices as measured by a mass assay (A and C) and the [^{3}H]adenine-prelabeling technique (B and D). (C) The mass data in A have been replotted with the basal cAMP level subtracted from each stimulated value. (D) The [^{3}H]adenine-prelabeling data in B have been replotted with the basal cAMP level subtracted from each stimulated value. Each point is the mean ± SEM of 7 experiments using tissue pooled from 2–3 animals for each experiment.

to basal, whereas forskolin (12 μM) caused a 5.3-fold increase (6.2 ± 0.67%). In the presence of both forskolin (12 μM) and oxo-M (100 μM), cAMP values were increased 8.3-fold (9.7 ± 0.74%) relative to basal. The effects of oxo-M and forskolin on cAMP accumulation were not affected by the addition of 1 μM tetrodotoxin (data not shown).

A comparison of the data in panels A and B of Fig. 1 shows that, when expressed relative to basal, the cAMP measurements with the [^{3}H]adenine-prelabeling technique are much larger than the corresponding measurements with the mass assay. This difference may be due to a relatively larger basal measurement with the mass assay. Thus, when the basal value is subtracted from the measurements made in the presence of the various drugs, there is much closer agreement between the two methods (see Fig. 1, C and D). Furthermore, when the data shown in panels C and D of Fig. 1 were expressed relative to the effect of forskolin rather than to basal, there were no significant differences between the two methods with regard to the effect of oxo-M by itself ($P > 0.1$) and its potentiation of the forskolin response ($P > 0.1$). The [^{3}H]adenine-prelabeling technique was used to characterize cAMP accumulation further because this method yielded lower basal values, which enabled a more accurate characterization of the response to oxo-M.

Since the experiments described above were done in the presence of the phosphodiesterase inhibitor IBMX, it was of interest to examine the effects of forskolin and oxo-M on cAMP accumulation in the absence of IBMX (Fig. 2). Consequently, additional experiments were run simultaneously in both the presence and absence of IBMX. In the presence of IBMX (0.5 mM), forskolin (25 μM) increased cAMP production 7.1 ± 0.40-fold over basal (0.9 ± 0.06%) and oxo-M (10 μM) caused a 2.2 ± 0.15-fold increase. When forskolin and oxo-M were combined, basal levels were increased 13.8 ± 1.59-fold. In the absence of IBMX, basal levels of cAMP accumulation decreased to 0.6 ± 0.07%. Forskolin (25 μM) and oxo-M (10 μM) stimulated cAMP accumulation 2.6 ± 0.15- and 1.4 ± 0.10-fold over basal, respectively, and their combination caused a 5.0 ± 0.58-fold increase with respect to basal, demonstrating that the mechanism for these responses is independent of phosphodiesterase activity. Since measurements of [^{3}H]cAMP were greater in the presence of IBMX, we included it in the remainder of our experiments.

The effects of various concentrations of oxo-M on cAMP accumulation in the rat lung are shown in Fig. 3. Oxo-M stimulated cAMP production in a concentration-dependent manner with an EC_{50} value of 4.2 μM and a maximal effect of 2.4 ± 0.39-fold over basal. When forskolin (25 μM) was combined with oxo-M at various concentrations, it potentiated the response elicited by oxo-M. This potentiation was more than additive since forskolin only increased cAMP accumulation 5.9 ± 2.2-fold over basal by

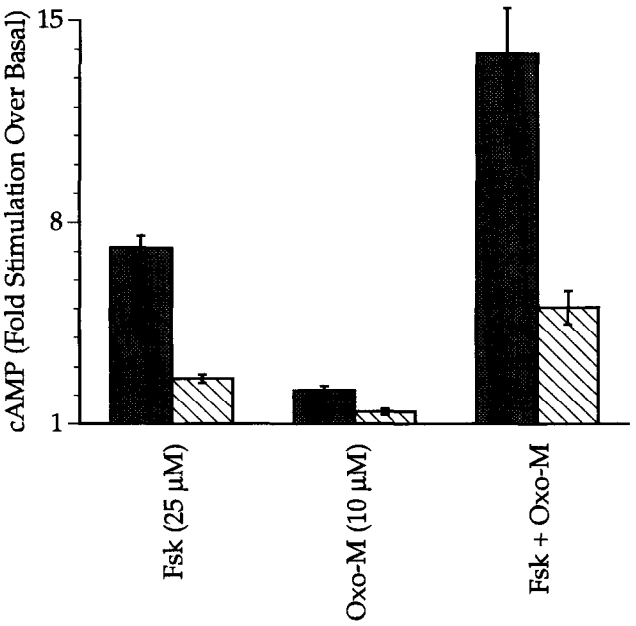

FIG. 2. Effects of oxo-M, forskolin (Fsk), and their combination on cAMP accumulation in rat peripheral lung slices in the presence (shaded bars) and absence (hatched bars) of IBMX (0.5 mM). All data are expressed relative to basal cAMP levels. Each point is the mean ± SEM of 4 experiments using tissue pooled from 2 animals for each experiment.

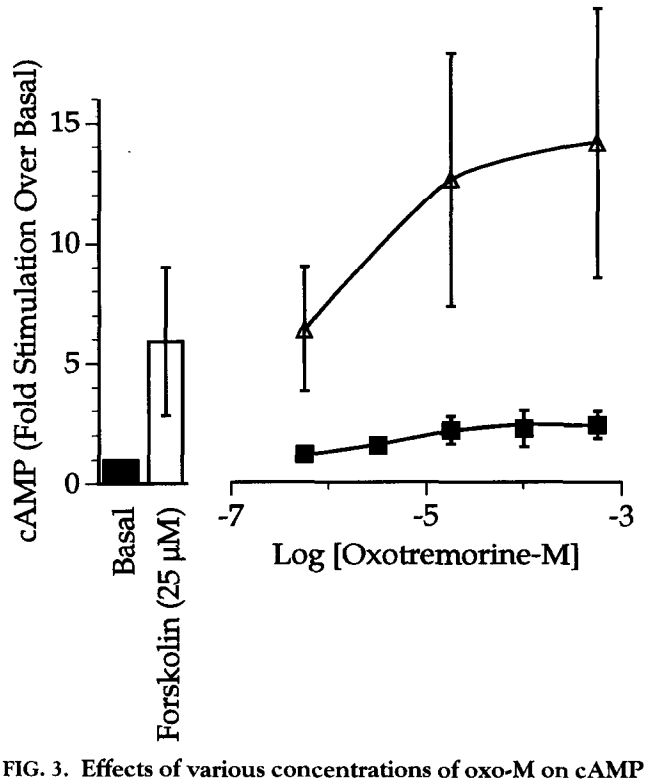

FIG. 3. Effects of various concentrations of oxo-M on cAMP accumulation in the absence (■) and presence (△) of forskolin (25 μM) in rat peripheral lung slices. All data are expressed relative to basal cAMP (solid bar). Effects of forskolin in the absence of oxo-M (open bar) were also measured. Basal cAMP conversion was 0.9 ± 0.09%. Each point is the mean ± range of two experiments using tissue pooled from two animals for each experiment.

itself, but the maximal effect of oxo-M was increased to 14 ± 4.0-fold when both drugs were combined.

The effects of various concentrations of forskolin on cAMP accumulation are shown in Fig. 4. Forskolin stimulated cAMP production in a concentration-dependent manner with an EC_{50} value of 4.5 μM and a maximal effect of 4.2 ± 0.37-fold over basal. This response was potentiated by oxo-M (10 μM) which increased the maximal response of forskolin to 9.4 ± 0.83-fold over basal. The effect of the two drugs combined was more than additive since oxo-M by itself was only able to increase cAMP accumulation 1.7 ± 0.10-fold over basal (see Fig. 5).

Additional agents known to stimulate adenylyl cyclase were screened to determine whether oxo-M (10 μM) potentiated their effects on cAMP accumulation (Fig. 5). For comparison, the potentiating effect of oxo-M on forskolin-stimulated cAMP accumulation is also shown in Fig. 5. Histamine (3 μM) had no significant effect on cAMP accumulation by itself; consequently, in the presence of both histamine and oxo-M, the level of cAMP was the same as that measured with oxo-M by itself (1.7 ± 0.10-fold over basal). Isoproterenol (0.1 μM) caused a 5.0 ± 0.54-fold increase in cAMP over basal, and this effect was increased significantly by oxo-M to 5.6 ± 0.58 ($P < 0.01$) in an additive manner. An additive increase by oxo-M was also observed with PGE_2 (10 μM). When used alone, PGE_2 significantly stimulated cAMP 1.6 ± 0.23-fold over basal ($P < 0.02$), and when combined with oxo-M, cAMP levels increased to 2.5 ± 0.29-fold over basal. The effect of oxo-M and PGE_2 was significantly greater than the effect of PGE_2 by itself ($P < 0.01$).

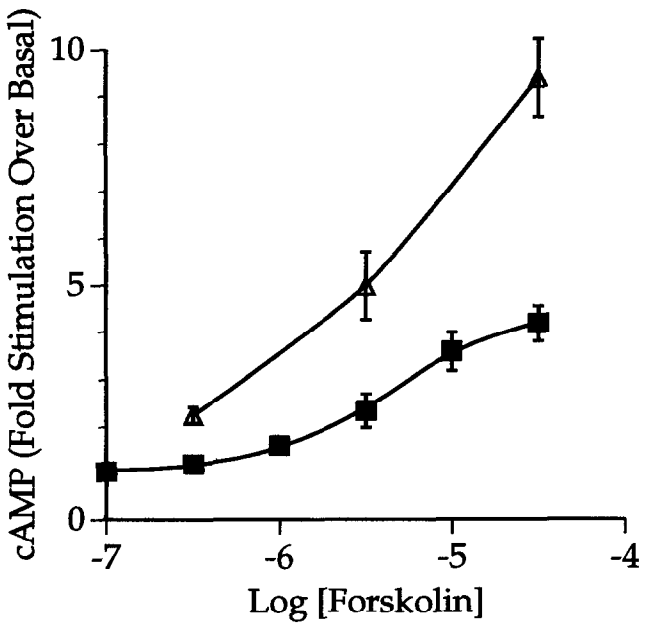

FIG. 4. Effects of various concentrations of forskolin on cAMP accumulation in the absence (■) and presence (△) of oxo-M (10 μM) in rat peripheral lung slices. All data are expressed relative to basal cAMP conversion which was 1.02 ± 0.18%. Each point is the mean ± SEM of 4 experiments using tissue pooled from 2 animals for each experiment.

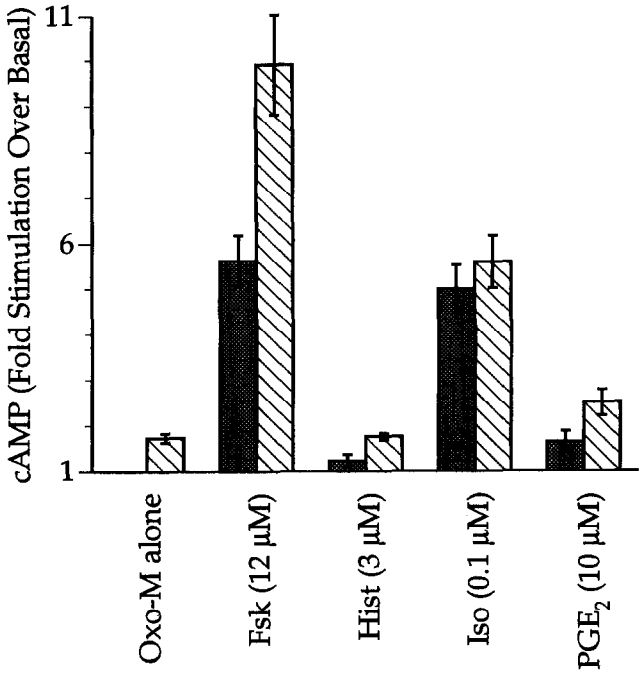

FIG. 5. Effects of forskolin (Fsk), histamine (Hist), isoproterenol (Iso), and prostaglandin E_2 (PGE_2) on cAMP accumulation in the absence (shaded bars) and presence (hatched bars) of oxo-M (10 μM) in rat peripheral lung slices. All data are expressed relative to basal cAMP levels. Each point is the mean ± SEM of 4–8 experiments using tissue pooled from 2–3 animals for each experiment.

A series of subtype selective muscarinic antagonists were tested for their ability to antagonize the potentiating effects of oxo-M on forskolin-stimulated cAMP accumulation to determine which subtype of the muscarinic receptor was mediating the response (Fig. 6). cAMP accumulation was measured in the presence of forskolin (25 μM) by itself, and combined with various concentrations of oxo-M. The oxo-M-mediated potentiation was expressed relative to the forskolin response. These measurements were repeated in the presence of a single concentration of antagonist. All of the antagonists caused parallel rightward shifts of the concentration–response curve of oxo-M. The dissociation constant (K_B) of each antagonist was estimated from the following equation:

$$K_B = \frac{[\text{antagonist}]}{CR - 1}$$

in which CR (Concentration Ratio) denotes the EC_{50} value of oxo-M measured in the presence of the antagonist divided by that measured in its absence. A summary of these results is provided in Table 1.

Radioligand Binding in CHO Cells

To provide a valid comparison between the K_B values of antagonists calculated in Table 1 and their respective bind-

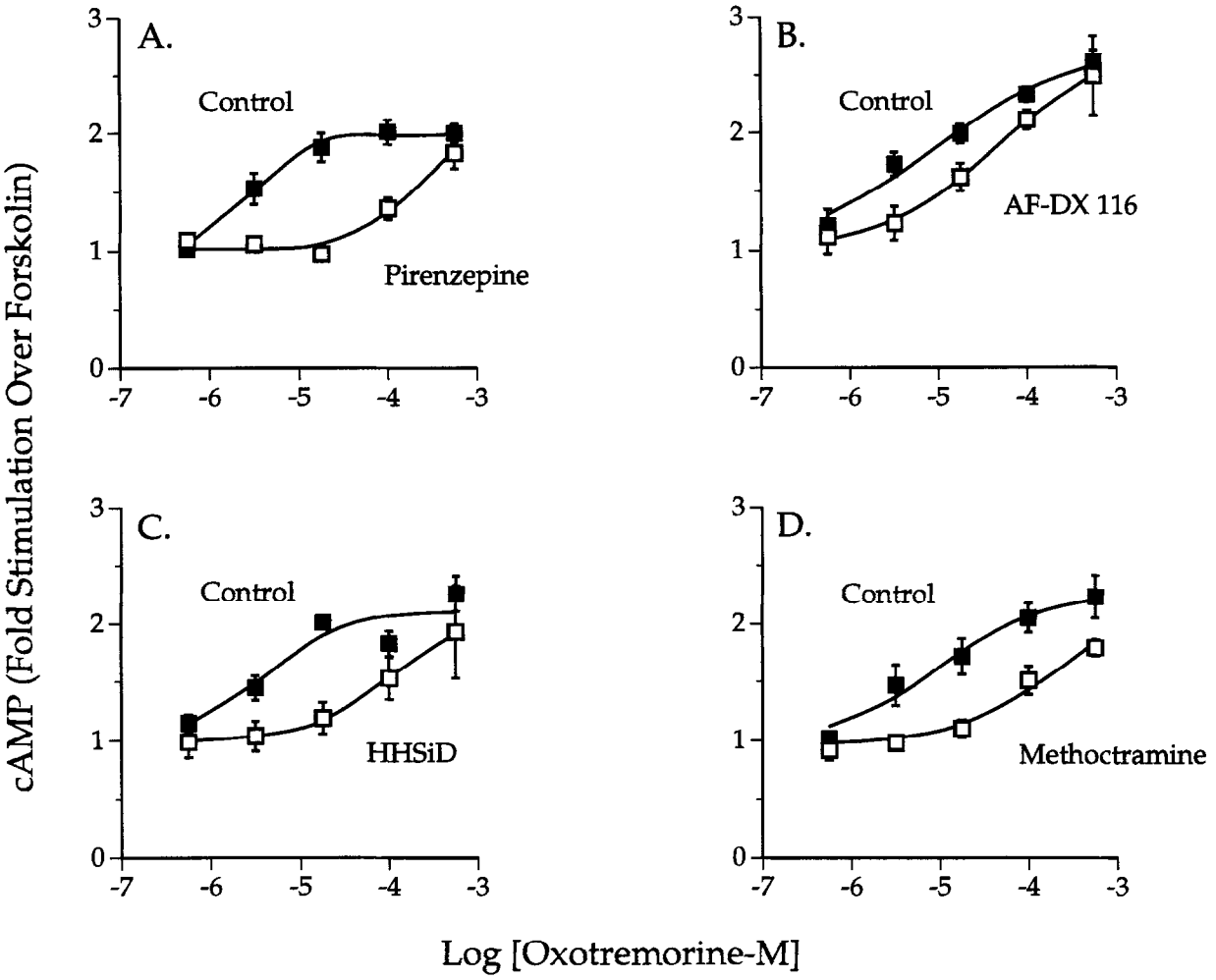

FIG. 6. Effects of various concentrations of oxo-M combined with forskolin (25 μM) on cAMP accumulation in the absence (■) and presence (□) of different muscarinic antagonists in rat peripheral lung slices. (A) 10 μM pirenzepine, (B) 10 μM AF-DX 116, (C) 1.0 μM HHSiD, and (D) 10 μM methoctramine. All data are expressed relative to the cAMP levels elicited by forskolin. Each point is the mean ± SEM of at least 4 experiments using tissue pooled from 3 animals for each experiment.

ing affinities (K_D values) at subtypes of the muscarinic receptor, we carried out antagonist/[^{3}H]NMS competition experiments in CHO cells transfected with the M_1–M_5 subtypes of the muscarinic receptor. These binding assays were run in a modified KRB buffer nearly identical to that used in the cAMP and phosphoinositide assays because it is known that ionic strength influences the binding affinities of muscarinic antagonists [40]. The estimated K_D values of

TABLE 1. pK_B values for antagonism of cAMP accumulation and phosphoinositide hydrolysis in the rat peripheral lung by various antagonists and binding affinities (pK_D) for the same antagonists at the human M_3 receptors transfected into CHO cells

	cAMP accumulation			Phosphoinositide hydrolysis			Radioligand binding
Antagonist	Concentration (μM)	Concentration ratio*	pK_B†	Concentration (μM)	Concentration ratio*	pK_B†	hM_3 pK_D‡
Pirenzepine	10	61.7	6.78 ± 0.09	1	7.7	6.80 ± 0.24	6.59 ± 0.03
AF-DX 116	10	13.6	6.09 ± 0.26	10	9.2	5.91 ± 0.11	6.10 ± 0.06
HHSiD	1	29.8	7.47 ± 0.21	1	43.7	7.63 ± 0.06	7.69 ± 0.06
Methoctramine	10	20.6	6.28 ± 0.20	10	6.0	5.70 ± 0.02	6.33 ± 0.04

* "Concentration ratio" denotes the EC_{50} value of oxo-M measured in the presence of the antagonist divided by that measured in its absence.
† Each pK_B is the mean ± SEM of 4–5 experiments using tissue pooled from 2–3 animals.
‡ Each pK_D is the mean ± SEM of 3 experiments.

antagonists at the M_1–M_5 muscarinic receptor subtypes are listed in Table 2, and the values at the M_3 subtype are also listed in Table 1 to facilitate comparison with the K_B values. Analysis of variance (see Materials and Methods) showed that there were no significant differences between the K_B values of antagonists measured by antagonism of the cAMP response and their respective binding affinities at the M_3 subtype ($F_{(3,23)} = 0.914$; $P = 0.457$). In contrast, there were significant differences between the K_B values from the cAMP response and the binding affinities estimated at the M_1 ($F_{(3,23)} = 16.9$; $P << 0.001$), M_2 ($F_{(3,23)} = 35.7$; $P = << 0.001$), M_4 ($F_{(3,23)} = 18.4$; $P << 0.001$), and M_5 ($F_{(3,23)} = 10.9$; $P << 0.001$) subtypes of the muscarinic receptor. We conclude that the M_3 subtype mediates the potentiation in the cAMP accumulation elicited by forskolin in the rat peripheral lung.

Adenylyl Cyclase Activity

Adenylyl cyclase activity was measured in homogenate preparations of the peripheral lung to determine if cAMP accumulation was due to direct stimulation of adenylyl cyclase (Fig. 7). In the absence of drugs, basal adenylyl cyclase activity was estimated to be 6.3 ± 0.85 pmol · min^{-1} · mg $tissue^{-1}$. Oxo-M (100 μM) inhibited basal adenylyl cyclase activity by 15% ($P < 0.01$), whereas forskolin (100 μM) and isoproterenol (50 μM) caused a 1.5 ± 0.07- and 1.5 ± 0.05-fold increase relative to basal, respectively. When forskolin (100 μM) and oxo-M (100 μM) were combined, they caused a 1.5 ± 0.04-fold stimulation over basal which was not significantly different from the response of forskolin alone ($P > 0.1$). These data provide no evidence for a direct stimulation of adenylyl cyclase activity by oxo-M in the peripheral lung.

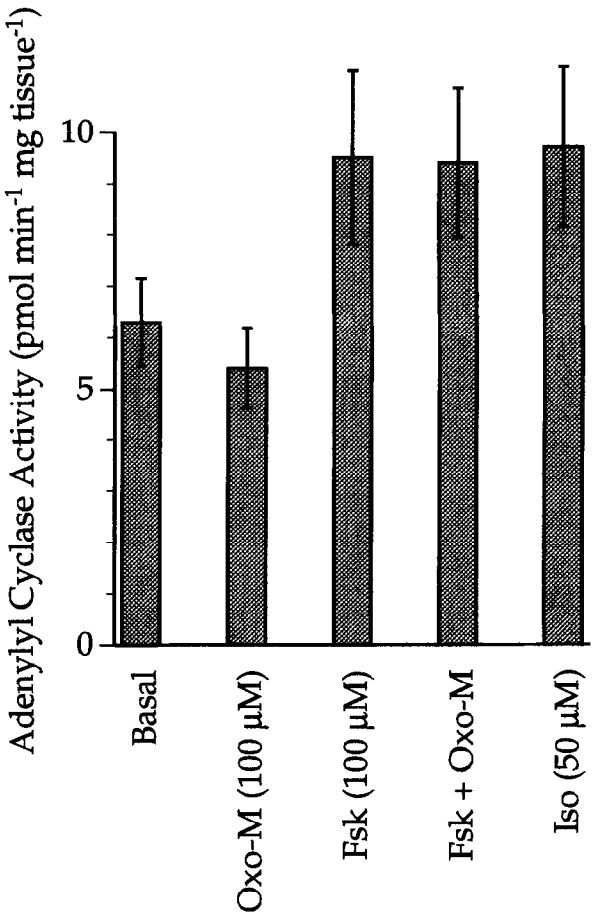

FIG. 7. Effects of oxo-M, forskolin (Fsk), their combination, and isoproterenol (Iso) on adenylyl cyclase activity in homogenates of the rat peripheral lung. Each point is the mean ± SEM of 4 experiments.

Phosphoinositide Hydrolysis

The M_1, M_3, and M_5 muscarinic receptor subtypes couple preferentially to the stimulation of phospholipase C-β, leading to the production of IP_3 and diacylglycerol [3]. To determine if this second messenger pathway was active in the rat peripheral lung slice preparation, muscarinic receptor-stimulated phosphoinositide hydrolysis was measured. The muscarinic agonist oxo-M stimulated the accumulation of labeled inositol phosphates with an EC_{50} value of 9.0 μM and a maximal effect of 1.9-fold relative to basal (Fig. 8). To determine which subtype of the muscarinic receptor mediated this effect, the same subtype-selective antagonists described above were tested for their ability to antagonize the phosphoinositide response. Oxo-M-mediated phosphoinositide hydrolysis was measured in the absence and presence of a single concentration of antagonist (see Fig. 8). All antagonists caused parallel rightward shifts of the concentration–response curves of oxo-M. Dissociation constants were calculated for each antagonist as described above (see equation for calculating K_B) and are listed in Table 1.

TABLE 2. pK_D Values for various muscarinic antagonists in CHO cells transfected with the human muscarinic receptor subtypes

Antagonist	hM_1	hM_2	hM_3	hM_4	hM_5
Pirenzepine	7.77 ± 0.03	5.96 ± 0.05	6.59 ± 0.03	7.23 ± 0.02	6.55 ± 0.06
AF-DX 116	6.24 ± 0.03	7.27 ± 0.05	6.10 ± 0.06	6.96 ± 0.12	5.29 ± 0.11
HHSiD	7.66 ± 0.08	6.74 ± 0.04	7.69 ± 0.06	7.65 ± 0.01	6.77 ± 0.01
Methoctramine	7.04 ± 0.03	7.29 ± 0.06	6.33 ± 0.04	7.02 ± 0.03	6.28 ± 0.06

Each pK_D is the mean ± SEM of 3 experiments.

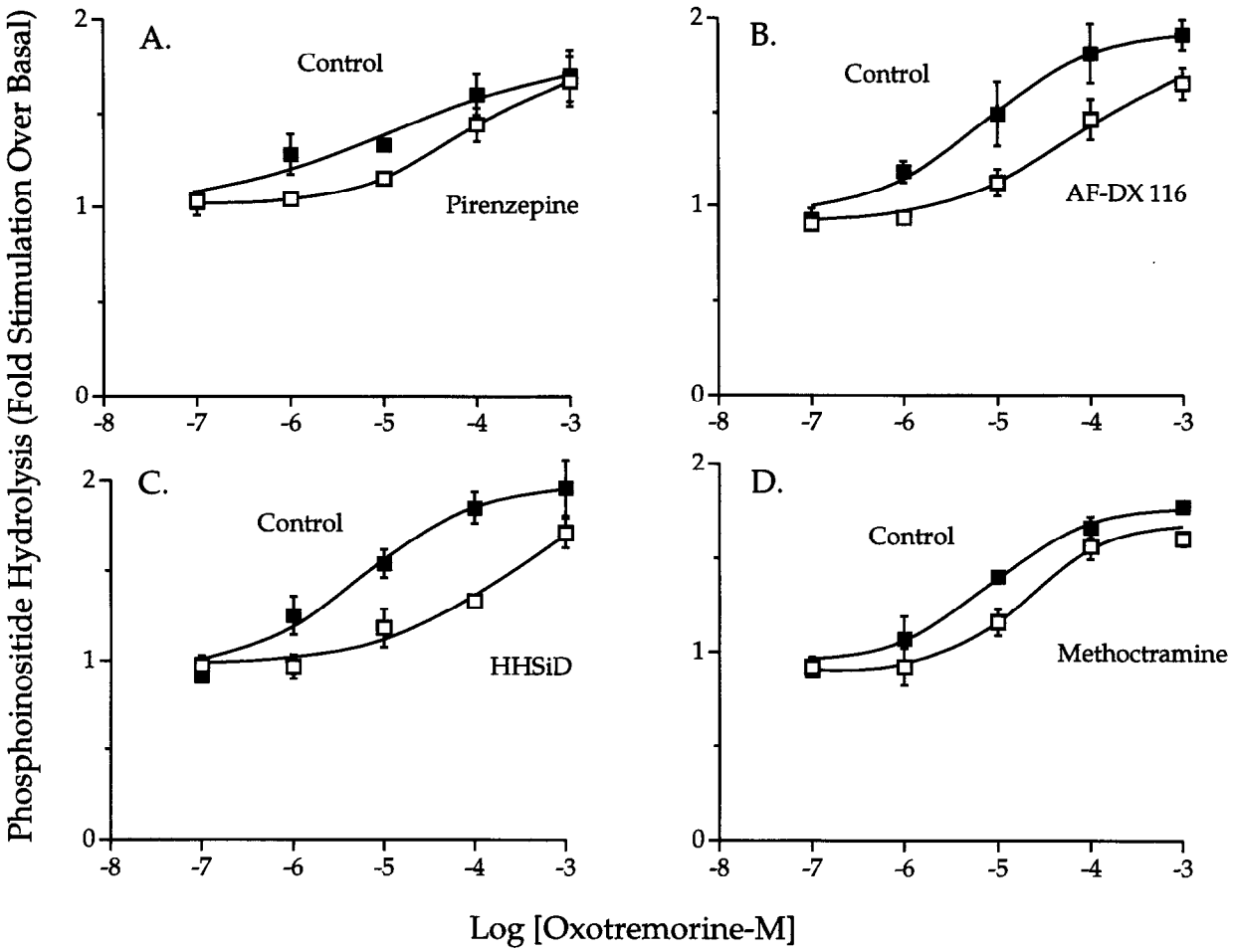

FIG. 8. Effects of various concentrations of oxo-M on phosphoinositide hydrolysis in the absence (■) and presence (□) of different muscarinic antagonists in rat peripheral lung slices. (A) 1 μM pirenzepine, (B) 10 μM AF-DX 116, (C) 1 μM HHSiD, and (D) 10 μM methoctramine. All data are expressed relative to basal inositol phosphate levels. Each point is the mean ± SEM of at least 4 experiments using tissue pooled from 3 animals for each experiment.

Analysis of variance showed that there were significant differences between the K_B values for antagonism of the phosphoinositide response and their respective binding affinities measured at each of the five subtypes of the muscarinic receptor; however, the K_B values agreed best with the M_3 subtype ($F_{(3,19)}$ = 8.04; P = 0.0012) as compared with the M_1 ($F_{(3,19)}$ = 49.6; $P << 0.001$), M_2 ($F_{(3,19)}$ = 106; $P << 0.001$), M_4 ($F_{(3,19)}$ = 49.3; $P << 0.001$), and M_5 ($F_{(3,19)}$ = 25.7; $P << 0.001$) subtypes. Analysis of variance was repeated omitting the K_B value of methoctramine, and it was found that there were no significant differences between the K_B values of the other three antagonists and their respective binding affinities at the M_3 subtype ($F_{(2,15)}$ = 1.96; P = 0.18), whereas there were differences at the M_1 ($F_{(2,15)}$ = 24.5; $P << 0.001$), M_2 ($F_{(2,15)}$ = 79.6; $P << 0.001$), M_4 ($F_{(2,15)}$ = 27.7; $P << 0.001$), and M_5 ($F_{(2,15)}$ = 25.3; $P << 0.001$) subtypes. We conclude that the M_3 subtype mediates the phosphoinositide response in the peripheral lung and that our estimate of the pK_B of methoctramine in the phosphoinositide assay is probably erroneously low (5.70 ± 0.02) compared with those measured in the cAMP (6.38 ± 0.20) and binding (6.33 ± 0.04) assays.

Effects of EGTA on cAMP Accumulation

To investigate the dependency of forskolin- and oxo-M-stimulated cAMP accumulation on an influx of external calcium, we measured the effects of a 5-min preincubation with EGTA (2 mM) on cAMP accumulation (Fig. 9). In the absence of EGTA, forskolin (25 μM) and oxo-M (10 μM) stimulated cAMP accumulation 7.0 ± 0.33- and 2.2 ± 0.14-fold over basal (0.8 ± 0.08%), respectively. When combined, oxo-M potentiated the forskolin response, and increased cAMP levels 12.2 ± 0.55-fold over basal. In the presence of EGTA (2 mM), the forskolin response decreased to 2.8 ± 0.53-fold over basal (1.0 ± 0.04%) and the oxo-M response (0.9 ± 0.04%) was not significantly different from basal ($P > 0.05$). When measured in the presence of both oxo-M and forskolin, the cAMP response (3.0 ± 0.53-fold) was not significantly different from that mea-

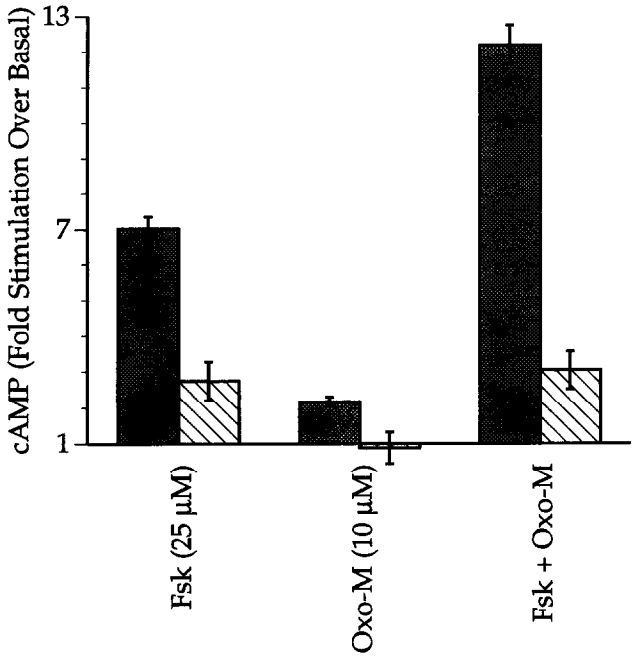

FIG. 9. Effects of forskolin (Fsk), oxo-M, and their combination on cAMP accumulation in rat peripheral lung slices in the absence (shaded bars) and presence (hatched bars) of EGTA (2 mM). The preincubation with EGTA was for 5 min. All data are expressed relative to basal cAMP levels. Each point is the mean ± SEM of 5 experiments using tissue pooled from 2 animals for each experiment.

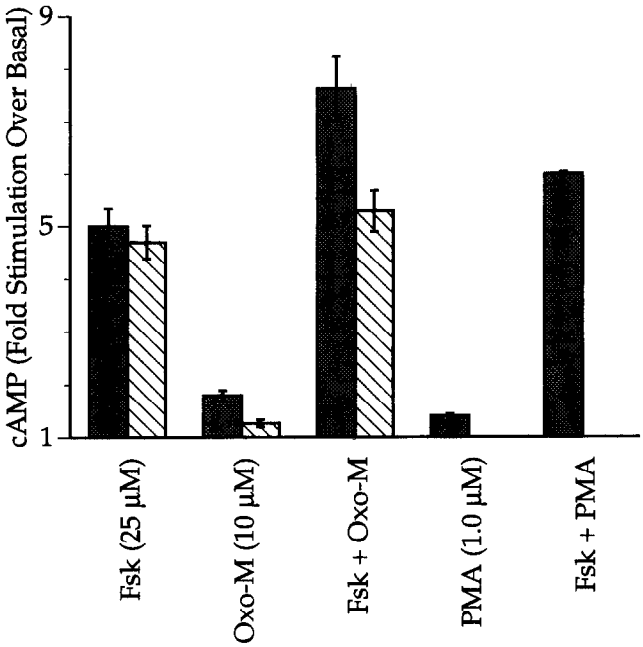

FIG. 10. Effects of forskolin (Fsk), oxo-M, their combination, PMA, and Fsk and PMA on cAMP accumulation in rat peripheral lung slices following a 1-hr preincubation in the absence (shaded bars) and presence (hatched bars) of PMA (1 μM). All data are expressed relative to basal cAMP levels. Each point is the mean ± SEM of 5 experiments using tissue pooled from 3 animals for each experiment.

sured in the presence of forskolin alone, indicating that EGTA prevented the potentiation of the forskolin response by oxo-M. These results demonstrate that external calcium is required for the effects of oxo-M whether alone or in combination with forskolin. In addition, at least part of the response to forskolin by itself requires external calcium.

Effects of PMA on cAMP Accumulation and Phosphoinositide Hydrolysis

To investigate the involvement of PKC in the oxo-M-mediated stimulation of cAMP accumulation, cAMP assays were done in the presence of PMA, a PKC activator. The first condition involved using PMA in place of oxo-M to see if PKC stimulation could mimic oxo-M in stimulating cAMP accumulation and potentiating forskolin (Fig. 10). PMA (1.0 μM) increased basal cAMP accumulation by 1.4 ± 0.03-fold, whereas oxo-M caused a 1.8 ± 0.09-fold increase with respect to basal (1.2 ± 0.10%). When PMA was combined with forskolin (25 μM), the response was nearly additive since there was a 14% increase in the response of forskolin alone, which was 5.0 ± 0.33-fold over basal. In contrast, oxo-M (10 μM) potentiated the forskolin response by 66%. These data suggest that PKC activation may result in a small stimulation of cAMP accumulation; however, it does not result in the potentiation of the forskolin response like that mediated by oxo-M.

The second condition with PMA involved measuring cAMP accumulation following a 1-hr preincubation with PMA (1.0 μM) in an attempt to down-regulate PKC (Fig. 10). In control tissue (preincubated for 1 hr without PMA), forskolin (25 μM) stimulated cAMP accumulation 5.0 ± 0.33-fold over basal (1.2 ± 0.10%), whereas oxo-M (10 μM) caused a 1.8 ± 0.09-fold increase. When combined, oxo-M and forskolin caused a 7.6 ± 0.61-fold increase in cAMP. This value corresponds to a 66% potentiation of the forskolin response by oxo-M. Pretreatment with PMA caused a small increase in basal levels of cAMP (1.8 ± 0.11%). When expressed relative to basal, the increase in cAMP elicited by forskolin (4.7 ± 0.31-fold) following preincubation with PMA was not significantly different from control ($P > 0.1$). In contrast, PMA treatment greatly reduced the response to oxo-M (1.3 ± 0.07-fold). When combined, forskolin and oxo-M stimulated cAMP 5.3 ± 0.39-fold, which corresponds to a 16% potentiation of the forskolin response by oxo-M. These data show that a lengthy incubation of the lung with PMA has no effect on the response to forskolin, but greatly inhibits the response to oxo-M by itself and its potentiation of the response to forskolin.

Phosphoinositide hydrolysis was also measured following preincubation with PMA to investigate whether PMA treatment affected this signaling pathway upstream from PKC. Following a 15-min preincubation with PMA (1 μM), oxo-M (9 and 100 μM) stimulated phosphoinositide hydrolysis 1.2 ± 0.05- and 1.4 ± 0.04-fold over basal, respectively (N = 4). These measurements were not signifi-

cantly different from control values (1.3 ± 0.08 and 1.4 ± 0.10, respectively). After a 1-hr preincubation with PMA (1 μM), oxo-M (9 μM) increased phosphoinositide hydrolysis 1.2 ± 0.09-fold over basal which was significantly lower than the control value (1.4 ± 0.08-fold) ($P < 0.05$). At the higher concentration of oxo-M (100 μM), phosphoinositide hydrolysis following PMA treatment (1.3 ± 0.13-fold) was not significantly different from control (1.5 ± 0.13-fold) ($P > 0.05$). These data show that lengthy pretreatment with PMA decreases oxo-M-stimulated phosphoinositide hydrolysis by 40–50%.

Effects of Indomethacin on cAMP Accumulation

It has been shown previously that muscarinic agonists stimulate arachidonic acid release and that some arachidonic acid metabolites can increase cAMP accumulation [11, 41, 42]. Therefore, we investigated whether indomethacin, a cyclooxygenase inhibitor, could inhibit the muscarinic response. Figure 11A shows the effects of indomethacin (1 μM) on the cAMP response to oxo-M and forskolin by themselves. Indomethacin had no effect on the basal levels of cAMP (data not shown). However, indomethacin caused a 92% inhibition of the cAMP accumulation stimulated by oxo-M (100 μM) and a 46% inhibition of that stimulated by forskolin (12 μM). In Fig. 11B, the effects of indomethacin (1 μM) on the interaction between oxo-M and forskolin are shown. It can be seen that indomethacin greatly reduced (60% inhibition) the potentiation of the forskolin response by oxo-M. These data show that arachidonic acid metabolites may be involved in cAMP accumulation mediated by oxo-M, forskolin, and their interaction.

Radioligand Binding in Rat Lung

Previous studies, using radioligand binding methods [25] as well as immunoprecipitation techniques [26], have demonstrated that the most abundant muscarinic receptor in the rat peripheral lung is the M_2 (80–90%). To confirm these studies, we carried out competitive binding experiments as described under Materials and Methods. Figure 12 shows the competitive inhibition of [^{3}H]NMS binding by various concentrations of pirenzepine in homogenates of the peripheral rat lung. Nonlinear regression analysis showed that the competition curve was consistent with a two-site model having high (K_H) and low (K_L) affinity dissociation constants. Pirenzepine bound to a majority of the sites (90%) with low affinity (K_L = 0.70 μM), whereas the remaining sites bound pirenzepine with high affinity (K_H = 0.035 μM). These results are consistent with previous findings demonstrating a large proportion of M_2 muscarinic receptors (K_D = 1.0 μM for pirenzepine, see Table 2) in the rat peripheral lung.

DISCUSSION

We have investigated the effects of a highly efficacious muscarinic agonist on cAMP accumulation in the rat peripheral lung, and our results show that oxo-M potentiates forskolin-stimulated cAMP production, but does not poten-

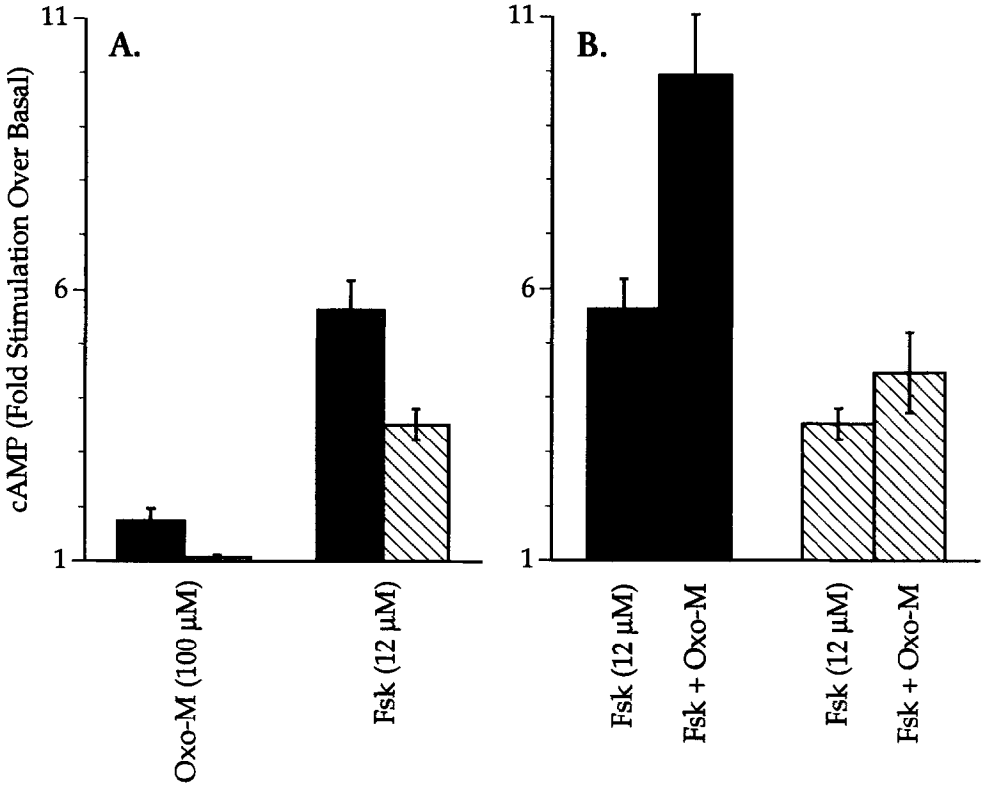

FIG. 11. (A) Effects of oxo-M and forskolin (Fsk) on cAMP accumulation in the absence (shaded bars) and presence (hatched bars) of indomethacin (1 μM) in rat peripheral lung slices. (B) Effects of Fsk alone and combined with oxo-M (100 μM) on cAMP accumulation in the absence (shaded bars) and presence (hatched bars) of indomethacin (1 μM). All data are expressed relative to basal cAMP levels. Each point is the mean ± SEM of 3 experiments using tissue pooled from 2 animals for each experiment.

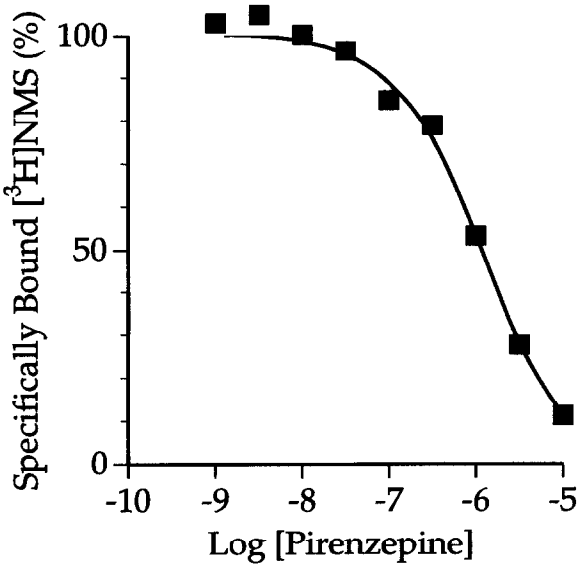

FIG. 12. Competitive inhibition of [^{3}H]NMS binding by pirenzepine in rat peripheral lung membranes. Specific binding of [^{3}H]NMS was measured at a radioligand concentration of 0.5 nM. Data are expressed as the percentage of the specific binding of [^{3}H]NMS measured in the absence of pirenzepine. Each point is the mean binding value of six experiments.

tiate the effects of isoproterenol, PGE_2, or histamine. This observation in peripheral lung contrasts with previous studies on the central airways where muscarinic agonists inhibit isoproterenol-stimulated cAMP accumulation [20, 23, 24]. Thus far, we are unable to specify which cell type is mediating the muscarinic response and how muscarinic subtypes are distributed among the different cell types in the peripheral lung. Moreover, the cells bearing the muscarinic receptors mediating the response may be different from those in which cAMP accumulates because arachidonic acid metabolites are probably involved in the response (see Fig. 11), and these metabolites may act on cells distinct from where arachidonic acid is released. However, it is certain that the cAMP response is not due to muscarinic stimulation of neurotransmitter release because the response was unaffected by tetrodotoxin.

The results from the mass assay and the [^{3}H]adenine-prelabeling technique were consistent in demonstrating that oxo-M potentiated forskolin-stimulated cAMP production. However, when cAMP accumulation was expressed relative to basal, the [^{3}H]adenine method gave larger estimates of cAMP than did the mass assay. The difference between the two methods is probably due to differences in the basal measurement of cAMP. One possible explanation is that there may be a basal pool of cAMP that does not get labeled with [^{3}H]adenine, giving rise to lower basal values with the [^{3}H]adenine-prelabeling assay.

To determine which subtype of the muscarinic receptor was mediating the cAMP response in the lung, we compared the K_B values of antagonists for blocking the response with their respective binding affinities (K_D values) at subtypes of the muscarinic receptor. For this comparison, we measured the K_D values of pirenzepine, AF-DX 116, methoctramine, and HHSiD in CHO cells transfected with the human M_1 through M_5 subtypes. These binding studies were carried out in a physiological buffer (KRB buffer) the same as that used in the cAMP assay because it is known that ionic strength influences muscarinic binding affinity [40]. Figure 13 shows that when the pK_B values for antagonism of the cAMP response are plotted against the corresponding binding affinity values for the M_3 receptor, a line of identity is formed, whereas the binding affinities at the other muscarinic subtypes are inconsistent with our pK_B values. Similarly, there is close agreement between our pK_B values for antagonism of the phosphoinositide response and the binding affinities at the M_3 subtype, with the two sets of values forming a line of identity (Fig. 14). Collectively, our results demonstrate the presence of functioning M_3 receptors in the rat peripheral lung.

Since both the cAMP and phosphoinositide responses are mediated by the M_3 receptor, then it follows that the antagonist K_B values for each response should be the same. To confirm this, we compared our antagonist pK_B values for the cAMP response to those for the phosphoinositide response. The pK_B values for pirenzepine, AF-DX 116, and HHSiD were in agreement (i.e. within 1.5-fold) between the two assays, demonstrating that the M_3 receptor subtype is mediating both responses (see Table 1). There was a modest discrepancy (3.8-fold) in the K_B value of methoctramine; nevertheless, analysis of variance showed no significant differences between the K_B values of antagonists determined from the two assays ($F_{(3,22)} = 2.58$; $P = 0.080$).

The results of competitive binding experiments with subtype selective antagonists indicate that approximately 80% of the muscarinic receptors in the rat peripheral lung are of the M_2 subtype [25]. Using antibodies selective for muscarinic subtypes, Wall *et al.* [26] confirmed the existence of a majority of M_2 receptors in the lung and detected small amounts of M_1 and M_3 receptors as well. Our binding experiments also confirm the great abundance of M_2 receptors in the rat lung as indicated by the large proportion (90%) of low affinity (K_L = 0.7 μM) pirenzepine binding sites. This low affinity is unique to the M_2 receptor since pirenzepine exhibits intermediate affinity (i.e. $K_D \approx 0.1$ μM) for the M_3, M_4, and M_5 subtypes and high affinity (i.e. K_D = 0.02 μM) for the M_1 subtype (see Table 2; see also Kashihara *et al.* [43]). It is interesting to note, therefore, that the cAMP and phosphoinositide responses characterized in this study are mediated by a very minor population of muscarinic receptors in the peripheral lung.

The mechanism for the small increase in cAMP elicited by oxo-M alone is unresolved at this point; however, it is probably not due to direct stimulation of adenylyl cyclase because oxo-M caused a small inhibition of adenylyl cyclase activity in a homogenate preparation. This inhibitory effect is probably mediated by the M_2 receptor which is known to inhibit adenylyl cyclase. Presumably, the small M_2-

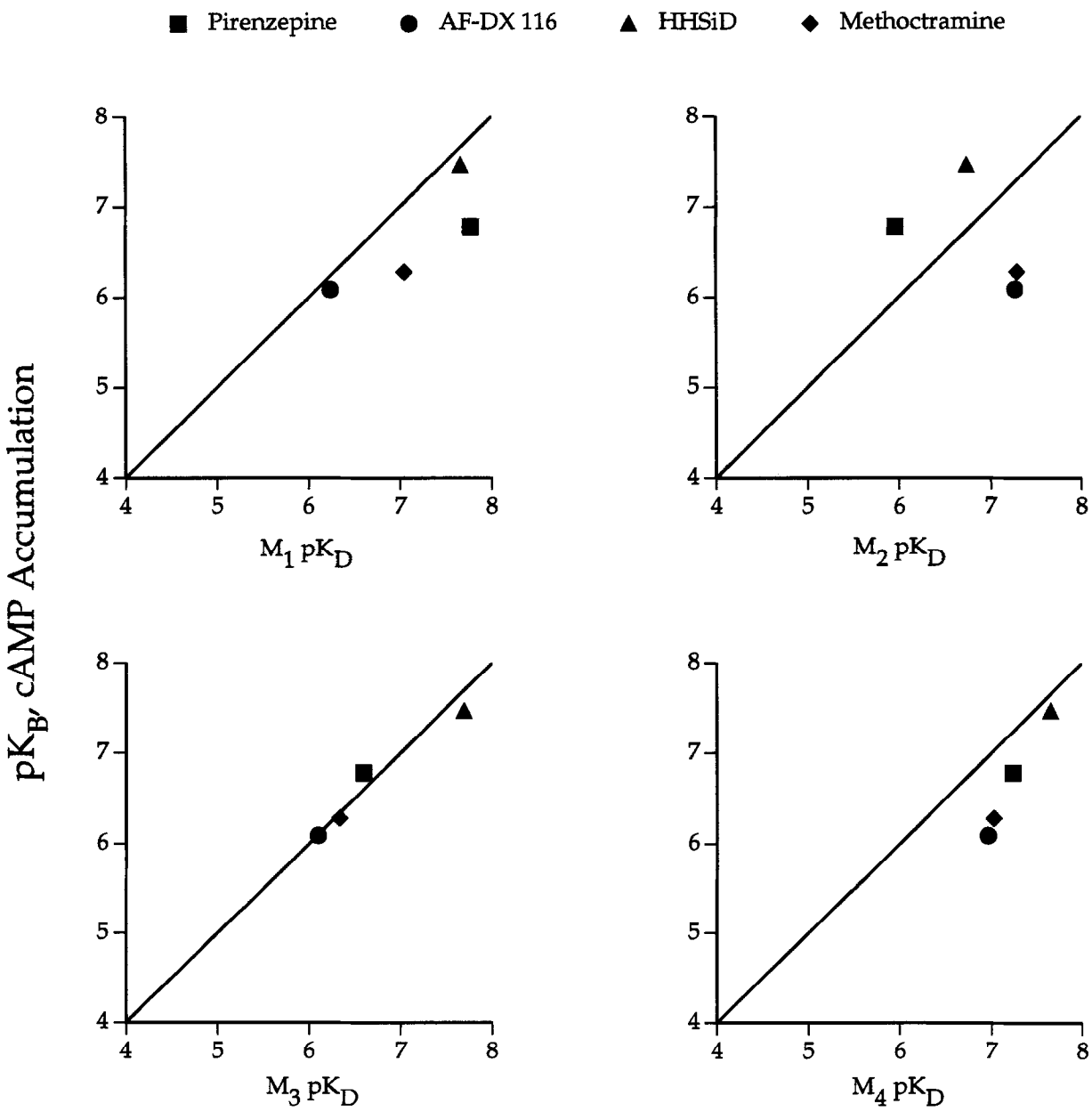

FIG. 13. Comparison of the pK_B values of antagonists for blocking cAMP accumulation in the rat peripheral lung (y-axis) with their respective binding affinities (pK_D) at the M_1–M_4 receptors (x-axis). The solid line denotes the line of identity (y = x), and has been inserted for comparative purposes. The pK_D values for pirenzepine, methoctramine, HHSiD, and AF-DX 116 are from Table 2.

mediated inhibition of adenylyl cyclase is overcome in the intact cell by a larger M_3-mediated increase in cAMP accumulation. It seems likely that the cAMP response to oxo-M by itself is mediated by arachidonic acid metabolites because it is blocked by the cyclooxygenase inhibitor indomethacin. In previous studies, muscarinic receptors have been shown to stimulate phosphoinositide hydrolysis and arachidonic acid release by varying mechanisms [9–11, 41, 44–47]. If the oxo-M-mediated cAMP response is caused by arachidonic acid metabolites, it is unclear whether this response is downstream from M_3-stimulated phosphoinositide hydrolysis or whether the response represents a parallel mechanism. Since the response was blocked by chelation of external calcium, it appears that the internal calcium released by an oxo-M-mediated increase in IP_3 is insufficient to generate cAMP accumulation. Nevertheless, this relatively small IP_3-mediated release of intracellular calcium could trigger a large influx of calcium by various mechanisms to activate phospholipase A_2. Therefore, these data are consistent with the hypothesis that the response may be due to arachidonic acid metabolites. Regardless of the source of arachidonic acid, its rapid conversion to metabolites could cause an activation of adenylyl cyclase through a variety of G_S-linked eicosanoid receptors. This phenom-

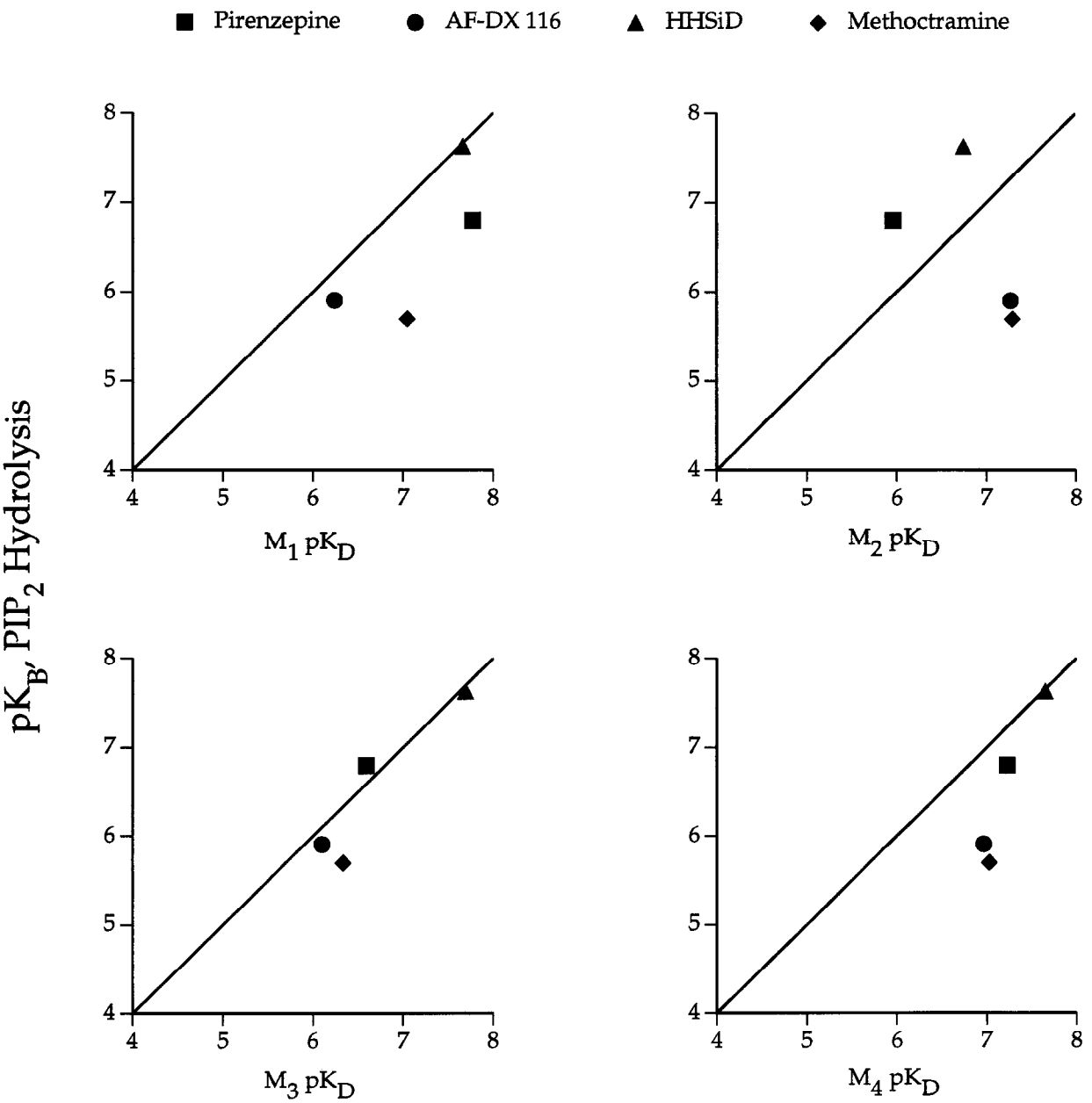

FIG. 14. Comparison of the pK_B values of antagonists for blocking phosphoinositide hydrolysis in the rat peripheral lung (y-axis) with their respective binding affinities (pK_D values) for the M_1–M_4 subtypes of the muscarinic receptor (x-axis). The solid line indicates the line of identity (y = x), and has been inserted for comparative purposes. The pK_D values of the antagonists are from Table 2.

enon has been demonstrated in the bovine ciliary muscle [41] and in canine parietal cells [42] following exposure to a muscarinic agonist.

The effect of EGTA on the oxo-M-mediated increase in cAMP also raises the possibility that a calcium-dependent isoform of adenylyl cyclase is mediating the response. However, since indomethacin completely blocked the response elicited by oxo-M, it is evident that oxo-M is not directly stimulating adenylyl cyclase via elevated calcium levels. It is conceivable that oxo-M may be stimulating the production of arachidonic acid metabolites that increase cAMP accumulation through a calcium-dependent isoform of adenylyl cyclase, but this has not been proven.

Arachidonic acid metabolites may also be involved in part of the forskolin-stimulated cAMP response because it was attenuated by indomethacin. It has been shown that arachidonic acid release can be augmented by cAMP [46]. Therefore, when forskolin directly stimulates adenylyl cyclase, a positive feedback phenomenon may occur in which the resulting cAMP formation triggers the release of arachidonic acid, causing the formation of metabolites such as prostaglandins that stimulate the formation of more cAMP.

The effects of EGTA on the forskolin response are consistent with this mechanism as well since arachidonic acid release may be indirectly dependent on calcium release. Likewise, the response may be partially due to the presence of a calcium-dependent isoform of adenylyl cyclase.

At the present time the mechanism for the potentiating effect of oxo-M on forskolin-stimulated cAMP accumulation is unclear. One possibility is that when forskolin binds to adenylyl cyclase, it sensitizes the enzyme to G_S so that the arachidonic acid metabolites generated by oxo-M can elicit an amplified cAMP response. A synergistic interaction between recombinant GTPγS-$G_{s\alpha}$ and forskolin has been demonstrated in homogenates of cells transfected with type II, IV, V, and VI adenylyl cyclase [48–50], and type II, V, and VI adenylyl cyclases have been found in the lung [51]. This mechanism can explain why oxo-M only potentiated the cAMP response to forskolin and not those elicited by isoproterenol and PGE_2. This mechanism also predicts that forskolin should interact synergistically with other agonists that signal through G_S in the lung. Interestingly, we have found that the cAMP response to forskolin and isoproterenol in the lung is much greater than the summation of their individual effects (data not shown). Further work is needed to sort out this mechanism.

Our studies with PMA showed that this PKC activator was unable to mimic oxo-M in potentiating forskolin-stimulated cAMP accumulation; however, a prolonged pretreatment with PMA inhibited muscarinic-stimulated phosphoinositide hydrolysis and prevented the muscarinic potentiation of cAMP accumulation. These results can be rationalized by assuming that PKC does not mediate the muscarinic-stimulated cAMP response; rather, the response may be downstream from IP_3 calcium release, or it may represent a parallel pathway that is also inhibited by PKC following excessive activation with PMA.

In summary, we have shown that a muscarinic agonist potentiates forskolin-stimulated cAMP accumulation and stimulates phosphoinositide hydrolysis in the rat peripheral lung. Both of these responses are mediated by the M_3 subtype of the muscarinic receptor. The mechanism for the muscarinic-mediated increase in cAMP levels has not been determined as of yet; however, it appears to involve arachidonic acid metabolites.

This work was supported by N.I.H. Grants NS 26511 and NS 30882. F. J. E. is a recipient of a United States Public Health Service Research Career Development Award (NS01396) from the National Institute of Neurological Disorders and Stroke.

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