Biochemical Pharmacology, Vol. 35, No. 14, pp. 2389–2397, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

BLOCKADE OF N1E-115 MURINE NEUROBLASTOMA MUSCARINIC RECEPTOR FUNCTION BY AGENTS THAT AFFECT THE METABOLISM OF ARACHIDONIC ACID

MICHAEL MCKINNEY and ELLIOTT RICHELSON
Department of Pharmacology, Mayo Foundation, Rochester, MN 55905, U.S.A.

(*Received* 13 *August* 1985; *accepted* 10 *December* 1985)

Abstract—Inhibitors of arachidonate metabolism and perturbants of the oxidation–reduction state of the cell were employed to develop a pharmacologic profile for muscarinic receptor-mediated cyclic GMP formation in murine neuroblastoma cells (clone N1E-115). Several lipoxygenase inhibitors [eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), FPL 57231, FPL 55712, BW755c, propylgallate, and AA861] blocked the elevation of [^{3}H]cyclic GMP induced by muscarinic receptor activation. The cyclooxygenase inhibitors indomethacin and ibuprofen were two orders of magnitude less potent in blocking the muscarinic receptor-mediated [^{3}H]cyclic GMP response than in blocking cyclooxygenase in other systems. ETYA and NDGA did not affect the muscarinic inhibition of the prostaglandin E_1-mediated increases in [^{3}H]cyclic AMP levels in N1E-115 cells. ETYA did not have a reproducible effect on the muscarinic receptor-induced release of inositol phosphates. Thus, these lipoxygenase inhibitors appeared to be selective for the effector system coupled to the low-affinity muscarinic agonist–receptor conformation, i.e. that which induces cyclic GMP formation. Other effective inhibitors of the cyclic GMP response were methylene blue, catalase, bromphenacyl bromide, retinal, dithiothreitol, quinacrine, and oxidized glutathione. The antioxidant α-tocopherol in the concentration range of 100 μM to 1 mM potentiated the receptor response. Arachidonic acid itself was an inhibitor of the muscarinic receptor-mediated cyclic GMP response (IC_{50} = 45 μM). Linoleic acid and oleic acid were less potent (IC_{50} = 130 and 190 μM, respectively), and stearic acid was ineffective. When arachidonic acid was air-oxidized, its inhibitory potency was increased 10-fold. Most but not all of the spontaneously-produced oxidative metabolites, separable by reverse-phase high pressure liquid chromatography, were inhibitory to the receptor response. Enzymatically synthesized 12-hydroxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid inhibited the muscarinic receptor [^{3}H]cyclic GMP response, with IC_{50} values of 17 and 8 μM respectively. Catalase was effective in blocking the muscarinic cyclic GMP response (IC_{50} = 5 μM) while having no effect on either the muscarinic receptor-induced inositol phosphate release or the reduction of cyclic AMP levels. Thus, the effector system for increasing cyclic GMP in these cells displays many of the expected characteristics for the involvement of a lipoxygenase or a related enzyme that oxidatively metabolizes arachidonate in order to activate the guanylate cyclase. A number of pharmacologic agents differentiate between this effector system and those to which the muscarinic receptor couples in order to effect cyclic AMP decreases or the release of inositol phosphates.

Muscarinic receptors of N1E-115 murine neuroblastoma cells exist in two subclasses, differentiated by their binding affinities for agonists or for the antagonist pirenzepine [1–3]. The data indicate that these subclasses mediate different cyclic nucleotide responses in N1E-115. Thus, a high-affinity agonist–receptor complex appears to be involved in the muscarinic inhibition of hormone-induced cyclic AMP elevation, with pirenzepine blocking this response with low potency. Most muscarinic agonists effectively activate this subclass of receptors. Conversely, a low-affinity agonist–receptor complex seems to be responsible for the elevation of cyclic GMP levels in N1E-115 cells. Only selected muscarinic agonists ("acetylcholine-like") are efficacious in mediating cyclic GMP stimulation, and pirenzepine inhibits this response with high potency, similar to its effects on the so-called M_1 type of muscarinic receptor (low nanomolar). Though the weight of the evidence supports the hypothesis of "separate sites/separate responses", additional biochemical and pharmacologic data that support or reject this hypothesis are desirable.

In the experiments described in this paper, in contrast to the previous studies with receptor agents, we have focused on the nature of the effectors involved in mediating the responses. Our recent work indicated that the effector for the elevation of cyclic GMP involves the metabolism of arachidonic acid. Thrombin, an agent that induces lipid metabolic events in platelets, stimulates cyclic GMP formation in N1E-115 cells [4]. Lipoxygenase inhibitors, but not cyclooxygenase inhibitors, block receptor-mediated elevation of cyclic GMP, and arachidonic acid is released [5]. This suggests that the effector for this response employs a mechanism of arachidonic acid release and metabolism to produce a hydroperoxide that activates guanylate cyclase, an hypothesis that is all the more attractive because of previous findings that this enzyme in various systems is activated by fatty acids, fatty acid peroxides, or hydroxyl radicals [6–8].

* Address all correspondence to: Dr. Michael McKinney, Abbott Laboratories, Dept. 47W, Bldg. AP10, North Chicago, IL 60064.

We extended our previous work to include the evaluation of a range of agents, including lipoxygenase inhibitors, cyclooxygenase inhibitors, and metabolic perturbants, for their abilities to affect receptor-mediated cyclic GMP formation. Further, several of these agents were tested against the muscarinic receptor-mediated inhibition of cyclic AMP formation and the muscarinic receptor-stimulation of inositol phosphate release. The findings indicate that the cyclic GMP response is unique in that it is selectively blocked by agents that prevent or interfere with the oxidative metabolism of arachidonic acid, probably by interacting at a lipoxygenase. Such agents appear to have no effect on the other two muscarinic responses.

MATERIALS AND METHODS

Cell culture and cyclic nucleotide assays. Murine neuroblastoma (clone N1E-115) cells were grown as described [9] in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Confluent cells from subcultures 9–14 were detached from flasks with Puck's D_1 solution, collected by low speed centrifugation, and washed in a physiological iso-osmolar PBS (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 25 mM Na_2HPO_4; pH adjusted to 7.4; osmolality adjusted to 340 ± 5 mOsm with sucrose). All bioassays were performed with N1E-115 cells suspended in this PBS. The assays for [^{3}H]cyclic GMP and [^{3}H]cyclic AMP levels in N1E-115 cells were performed as previously described [9, 10]. For testing the inhibitors in the [^{3}H]cyclic GMP assay, the muscarinic receptor was stimulated with 1 mM carbachol, a concentration giving a maximal response. For testing the inhibitors in the [^{3}H]cyclic AMP assay, the concentration of prostaglandin E_1 was 10 μM; carbachol at 1 mM was used to produce a muscarinic receptor-mediated inhibition of the [^{3}H]cyclic AMP response to the prostaglandin. Fatty acids and their analogues were generally added as sodium salts; care was taken to avoid excessive vortexing, as the resulting foaming and presumed oxidation of the lipids increased their inhibitory potencies. FPL 55712 was sparingly soluble in PBS, and in several assays a lack of inhibition by it was presumed to be due to its precipitation. This problem was not encountered with FPL 57231. Propylgallate, BW755c and α-tocopherol were added to the well in ethanol, which was evaporated before adding cells. AA861 and stearic acid were added as suspensions. Generally, the remaining drugs appeared to be soluble at the final concentration in PBS. Drugs were incubated with the cells for 30 min at 37° in a shaking water bath before the addition of agonists.

Assay for the release of [^{3}H]*inositol phosphates.* Cells in monolayer were labeled overnight in 10 ml of inositol-free medium (Grand Island Biological Co.; supplemented with 10% fetal bovine serum) with 40 μCi [^{3}H]inositol (14 Ci/mmole; Amersham) previously purified of polar impurities by chromatography on a Dowex anion exchange column. Prelabeled cells were detached with D_1, washed, and suspended in PBS (without lithium ion), distributed to 13 × 100 mm glass tubes, and incubated at 37° with various inhibitors for 30 min in a shaking water bath. After stimulation with 1 mM carbachol for 30 sec, the reaction was stopped by the addition of 750 μl of ice-cold chloroform–methanol (1:2) and vortexing. The phases were split by the addition of 250 μl [^{14}C]inositol-1-phosphate (approximately 1500 dpm) in water and 250 μl chloroform. After further vortexing, the tubes were centrifuged at 1500 rpm for 5 min in a low-speed centrifuge. An aliquot (600 μl) of the upper phase was transferred to another tube and mixed with 2 ml of water. The diluted sample was loaded on a column of AG-1 × 2 anion exchange resin (0.6 × 1.0 cm; Bio-Rad Laboratories, Richmond, CA), previously converted to the formate form. Free [^{3}H]inositol and [^{3}H]glycerophosphoinositol were eluted with 20 ml of 5 mM sodium tetraborate/60 mM ammonium formate. Total [^{3}H]inositol phosphates were eluted with 2 × 4 ml of 1 M ammonium formate/100 mM formic acid. Radioactivity was measured by liquid scintillation counting. Preliminary experiments showed that the carbachol-induced release of total inositol phosphates was sensitive to 10 μM atropine but insensitive to 10 μM *d*-tubocurarine, indicating that muscarinic receptors were involved. Time course studies showed that maximum release was attained by 15 sec and maintained for at least 2 min. The EC_{50} for carbachol in stimulating the inositol phosphate release was 16 μM.

Synthesis of arachidonic acid products and high-pressure liquid chromatographic procedures. Arachidonic acid was exposed to air as a thin film on glass for >24 hr, or it was oxidized chemically with $CuCl_2$ and H_2O_2 [11]. Usually a small amount of [^{14}C]arachidonic acid was added to allow measurement of the concentration of products. An example of a typical specific activity was 17 μCi/mmole. After extraction (in the case of chemical oxidation) the resulting material was dissolved in ethanol for injection into a Waters μBondapak C-18 reverse-phase HPLC column and eluted with methanol–water–acetic acid (75:25:0.0001). Detection was by absorbance at 254 nm. Fractions containing polar oxidation products were acidified with 0.1 N HCl and extracted with diethylether. The ether was evaporated, and the extracted material was reconstituted in concentrated form in ethanol. The oxidized derivative was introduced in a volume of 1 μl into the wells containing N1E-115 cells in a total volume of 300 μl; the same amount of carrier ethanol was added to control wells. This amount of ethanol had only a minor effect (no more than 20% reduction) on the muscarinic receptor-induced cyclic GMP stimulation. Alternatively, the sodium salt of the arachidonic acid metabolite was made by the addition of a slight excess of 0.01 N NaOH to the dried residue and diluting with water. Comparable results were obtained by either method of adding fatty acid derivatives.

[^{14}C]Hydroxyeicosatetraenoic acid ([^{14}C]15-HETE) was synthesized by the procedure of Graff [12], using soybean lipoxygenase. The specific activity varied from preparation to preparation; an example value was 59.5 μCi/mmole. [^{14}C]15-HETE was separated from untreated arachidonate on a column of Silicar CC-4. The product eluted from the

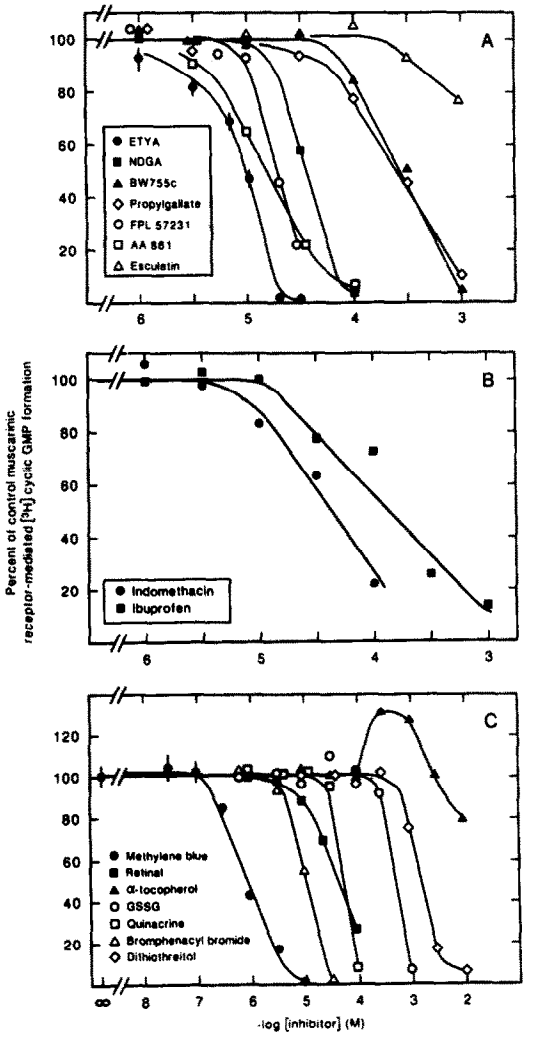

Fig. 1. Effects of various inhibitors on carbachol (1 mM)-stimulated muscarinic receptor-mediated cyclic GMP formation in N1E-115 murine neuroblastoma cells. (A) Inhibition by lipoxygenase inhibitors: ETYA, NDGA, BW755c, propylgallate, FPL 57231, AA861, and esculetin. (B) Inhibition by cyclooxygenase inhibitors: indomethacin and ibuprofen. (C) Inhibition by perturbants of cellular oxidation–reduction potential: methylene blue, retinal, α-tocopherol, oxidized glutathione (GSSG), quinacrine, bromphenacyl bromide, and dithiothreitol. The experiments shown are representative of two to seven experiments for each agent. IC_{50} values are shown in Table 1. See Materials and Methods for comments regarding solubility of certain of these agents.

silicic acid column was further purified by HPLC or TLC. At this point in the purification, two early peaks more polar than [^{14}C]15-HETE were evident in the profile; these were presumed to be 5,15-DiHETE and 8,15-DiHETE, as shown previously by other workers [13, 14], and were obtained for evaluation in the inhibition assays.

[^{14}C]12-HETE (specific activity 73.6 μCi/mmole) was synthesized with platelet lipoxygenase using the procedure of Sun [15] and purified by the same procedures described above for 15-HETE.

RESULTS

Inhibition of muscarinic receptor-mediated [^{3}H]*-cyclic GMP formation by arachidonic acid inhibitors and other metabolic inhibitors.* A wide variety of agents was screened for the ability to block muscarinic receptor-induced cyclic GMP formation in the N1E-115 neuroblastoma cells. Several experiments with lipoxygenase inhibitors are shown in Fig. 1A. Table 1 lists the IC_{50} values for the various agents tested. Eicosatetraynoic acid (ETYA) and AA861 were among the most potent of all the agents tested. These drugs inhibited the [^{3}H]cyclic GMP formed in response to 1 mM carbachol both with IC_{50} values averaging 10 μM. Nordihydroguaiaretic acid (NDGA) was about 2-fold less potent, having an IC_{50} of 19 μM. BW755c was much less potent, inhibiting the response to the muscarinic agonist with an IC_{50} value of 190 μM. The leukotriene inhibitors FPL 55712 and FPL 57231 blocked the receptor response with IC_{50} values of 20 and 24 μM respectively. Propylgallate was relatively impotent, having an IC_{50} of 560 μM. Esculetin was also ineffective, except at concentrations approaching 1 mM, in blocking [^{3}H]cyclic GMP formation in N1E-115 cells (Fig. 1A). Experiments with ETYA and NDGA indicated that their mechanism of inhibition of muscarinic receptor-mediated cyclic GMP formation was of the non-competitive type (data not shown). The K_i

Table 1. IC_{50} Values for some inhibitors of muscarinic receptor-mediated [^{3}H]cyclic GMP formation

Inhibitor (N)	IC_{50} (μM)
Methylene blue (3)	0.7 ± 0.1
Peak A (3)	4 ± 1
Catalase (3)	5 ± 1
15-HETE (7)	8 ± 1
AA861 (5)	10 ± 1
ETYA (6)	10 ± 3
Bromphenacyl bromide (2)	14 ± 4
12-HETE* (3)	17 ± 2
NDGA (7)	19 ± 3
FPL 55712† (3)	20 ± 3
FPL 57231 (5)	24 ± 4
Indomethacin (4)	30 ± 5
Retinal (5)	30 ± 6
Arachidonic acid (5)	45 ± 5
Quinacrine (4)	50 ± 7
Linoleic acid (3)	130 ± 40
Ibuprofen (3)	170 ± 30
Oleic acid (2)	190 ± 10
BW755c (5)	190 ± 20
Propylgallate (5)	560 ± 80
Dithiothreitol (3)	1500 ± 300
Oxidized glutathione (5)	3000 ± 1000

Averages ± S.E. or range (in μM) are shown. The inhibitors were incubated with N1E-115 neuroblastoma cells for 30 min at 37° at various concentrations before stimulating with 1 mM carbachol for 30 sec. The inhibitors were tested for the total number of replicates indicated (N), combined from two or more independent experiments.

* 12-HETE was tested in only one experiment due to the amount of material available, but the experiment was performed in triplicate.

† FPL 55712 occasionally did not inhibit, presumably due to difficulty in maintaining it in solution.

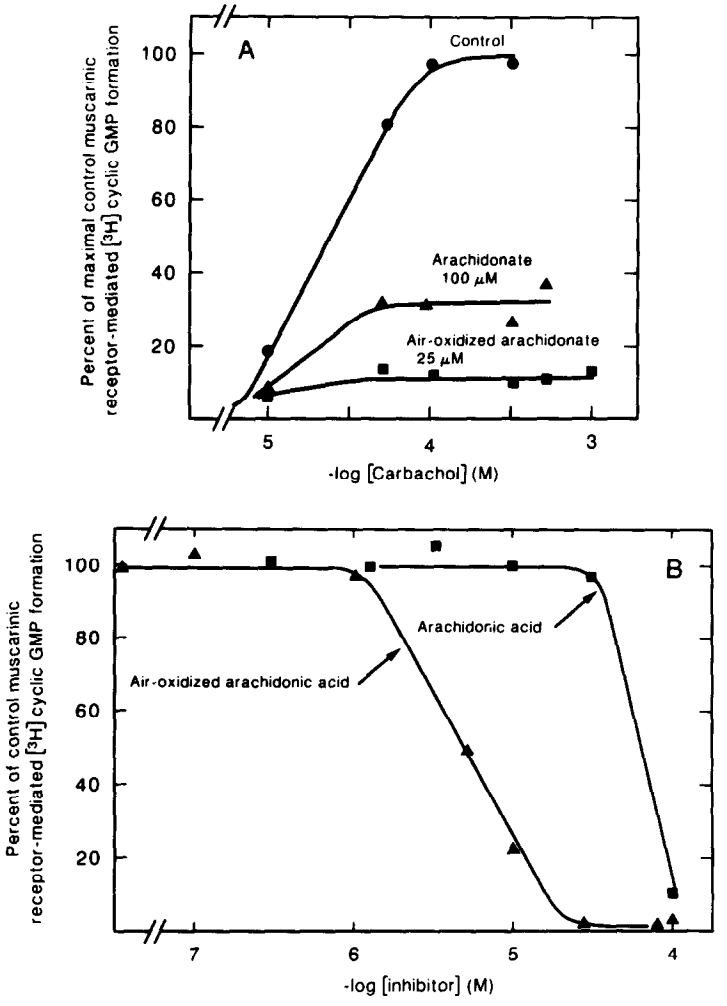

Fig. 2. Effect of arachidonate and air-oxidized arachidonate on muscarinic receptor-mediated cyclic GMP formation. (A) Effect on the carbachol concentration–response relation. Cells were incubated with 100 μM arachidonate or 25 μM oxidized arachidonate for 30 min prior to stimulation with various concentrations of carbachol. This type of experiment was performed at least four times with oxidized arachidonate and five times with arachidonate. (B) Concentration–inhibition curves for arachidonate and oxidized arachidonate. Various concentrations of the fatty acid preparations were incubated with N1E-115 cells for 30 min before stimulation with 1 mM carbachol. The IC_{50} values were 60 μM for arachidonic acid and 4 μM for oxidized arachidonate.

values for ETYA and NDGA, determined by the method of Dixon [16], were 5 and 11 μM respectively.

Indomethacin and ibuprofen were able to block [^{3}H]cyclic GMP increases induced by the muscarinic receptor (Fig. 1B), but at excessively high concentrations [IC_{50} values were 30 and 170 μM respectively (Table 1)], many times greater than what would be expected for the inhibition of cyclooxygenase. However, at these concentrations these drugs would be expected to inhibit lipoxygenase.

Several drugs able to change the oxidation–reduction state of the cell, or otherwise perturb arachidonic acid metabolism, were able to affect receptor-mediated [^{3}H]cyclic GMP formation (Fig. 1C). The most potent of the inhibiting agents were the oxidant methylene blue and the phospholipase A_2 inhibitor bromphenacyl bromide (see also Table 1). Methylene blue inhibited the response with an IC_{50} value of 0.7 μM. Bromphenacyl bromide inhibited cyclic GMP formation with an IC_{50} value of 14 μM. The IC_{50} values for the other agents tested in the experiments shown in Fig. 1C (and Table 1) were: retinal (30 μM), quinacrine (50 μM), dithiothreitol (1.5 mM) and oxidized glutathione (3 mM). Interestingly, α-tocopherol, a dietary essential lipid that is probably an important brain lipid antioxidant, potentiated the muscarinic receptor-mediated [^{3}H]cyclic GMP response at concentrations up to 1 mM (observed in two independent experiments; Fig. 1C). The results with the metabolic inhibitors and the antioxidants suggested that receptor-mediated activation of cyclic GMP formation in neural cells involves a lipid oxidation process and possibly the oxidation of sulfhydryl groups.

Inhibition of receptor-mediated [^{3}H]cyclic GMP formation by oxidized arachidonate metabolites. Arachidonic acid was found to be an inhibitor of the

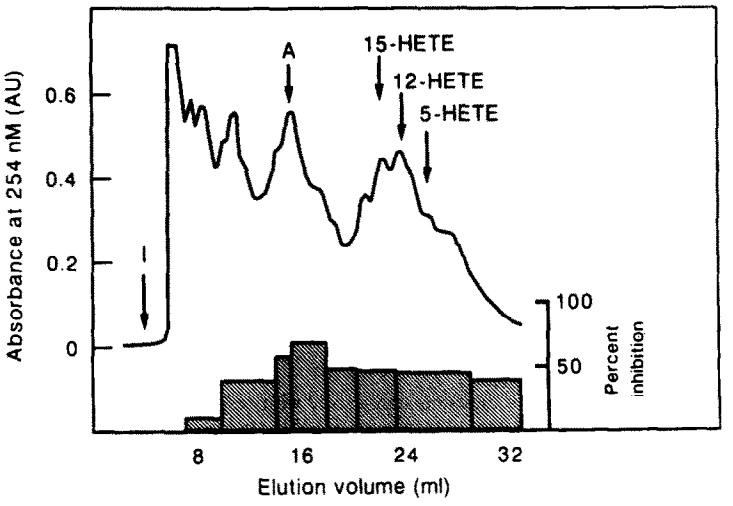

Fig. 3. Chromatographic and inhibitory profile for oxidized arachidonate material. Oxidized [^{14}C]arachidonate was subjected to isocratic reverse-phase high-pressure liquid chromatography. The column was an analytical Waters C-18 HPLC column. The mobile phase was methanol–water–acetic acid (75:25:0.0001) at a flow rate of 1 ml/min. The detector monitored absorbance at 254 nM. Fractions were collected, and the eluted material was extracted and tested for inhibition of 1 mM carbachol-stimulated muscarinic receptor-mediated cyclic GMP formation. For each fraction, the concentration of material derived from [^{14}C]arachidonate that was tested in the cyclic GMP assay was 0.7 μg. The assays were performed in triplicate. At least nine chromatograms were obtained showing this absorption profile. This experiment is one of two that tested all fractions for inhibition. Peak A was cut out and tested at least three further times.

response to the receptor (Fig. 2A). The IC_{50} value for unoxidized arachidonate was 45 μM (Fig. 2B and Table 1). Linoleic acid and oleic acid also inhibited the muscarinic receptor-mediated cyclic GMP response, but with IC_{50} values greater than 100 μM (see Table 1). Stearic acid at 100 μM did not block cyclic GMP formation (the response was 90 ± 4% of control, N = 4). These data suggest that the unsaturated nature of the fatty acid was important for inhibition.

When arachidonic acid was oxidized, either spontaneously or by chemical reaction with Cu^{2+} and H_2O_2, its potency was increased. Figure 2A compares the inhibition by a high concentration (100 μM) of unoxidized arachidonate with that by a 4-fold lower concentration of air-oxidized arachidonate. The latter preparation was considerably more effective in preventing cyclic GMP elevation. The concentration–inhibition curves (Fig. 2B) indicated that the potency of arachidonate was increased about 10-

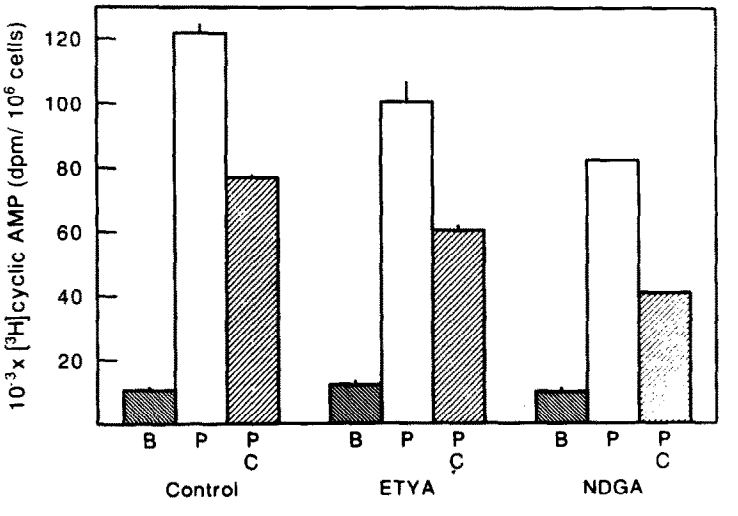

Fig. 4. Lack of effect of ETYA and NDGA on muscarinic receptor-mediated inhibition of prostaglandin E_1-stimulated cyclic AMP increases. ETYA (50 μM) and NDGA (50 μM) were incubated with N1E-115 cells in triplicate at 37° for 30 min before stimulating with prostaglandin E_1 (P; 1 μM) or the prostaglandin + carbachol (1 mM) (P + C). Basal levels are also shown (B). The degree to which carbachol inhibited the prostaglandin stimulation was not significantly different for all three sets of data. ETYA was tested in this type of assay three times and NDGA was tested once.

Table 2. Effects of ETYA and catalase on muscarinic receptor-mediated release of inositol phosphates

Experiment	[^{3}H]Inositol phosphates released* (dpm/million cells)	Response
Basal	38,000 ± 3,000	
Carbachol (1 mM)	57,000 ± 2,000	19,000 (100%)
+ ETYA (50 μM)	68,000 ± 5,000†	30,000 (157%)
+ Catalase (12 μM)	70,000 ± 4,000†	32,000 (169%)

ETYA (50 μM) and catalase (12 μM) were incubated for 30 min at 37° with N1E-115 cells prelabeled overnight with 40 μCi [^{3}H]inositol. Buffer ("basal") or 1 mM carbachol was added for 30 sec before termination of release and assay for released [^{3}H]inositol phosphates. Averages ± S.E. are shown. The assays were performed in quadruplicate.

* All responses were elevated signficantly with respect to basal, $P < 0.005$.

† The responses in the presence of ETYA and catalase were potentiated significantly with respect to carbachol alone: $P < 0.05$ (ETYA); and $P < 0.025$ (catalase). This particular experiment was performed twice with similar results. Two other experiments with ETYA indicated some inhibition of the response (see text). Catalase and ETYA had no effect on the basal release (not shown).

fold by air-oxidation. The reduction of the V_{max} of the carbachol-cyclic GMP concentration–response curves suggested that the mechanism of inhibition by arachidonic acid or oxidized arachidonate was non-competitive in nature. Other experiments showed that the inhibition by oxidized arachidonate was rapid ($t_{\frac{1}{2}} = 3$ min) and reversible (data not shown).

Oxidized [^{14}C]arachidonate was fractionated by HPLC, and the various peaks were tested for their relative potencies in inhibiting the muscarinic receptor response (Fig. 3). The profile of absorbance shown in Fig. 3 was mirrored by a similar profile of radioactive products, in a separate experiment, when the amount of radiolabeled arachidonate in each 1-ml fraction was counted (data not shown). Indicated in Fig. 3 are the retention volumes of standard monohydroxyeicosatetraenoic acids (HETEs). Not all of the oxidative metabolites of arachidonate were equally inhibitory. In the experiment shown in Fig. 3, each fraction was tested at a metabolite concentration of 0.7 μg per assay well (about 7 μM), based on the amount of radiolabel from the original [^{14}C]arachidonate found in the fraction. Material chromatographing in the area of the monoHETEs was inhibitory as was material with the retention characteristics of dihydroxylated derivatives (in the vicinity of peak A). Peak A was the most inhibitory area of the chromatogram (see Table 1). The most polar material (8–10 ml retention volume), by contrast, was not inhibitory.

Peak A of the air-oxidized material (Fig. 3) was cut from the chromatogram and rechromatographed on both reverse-phase and normal-phase HPLC

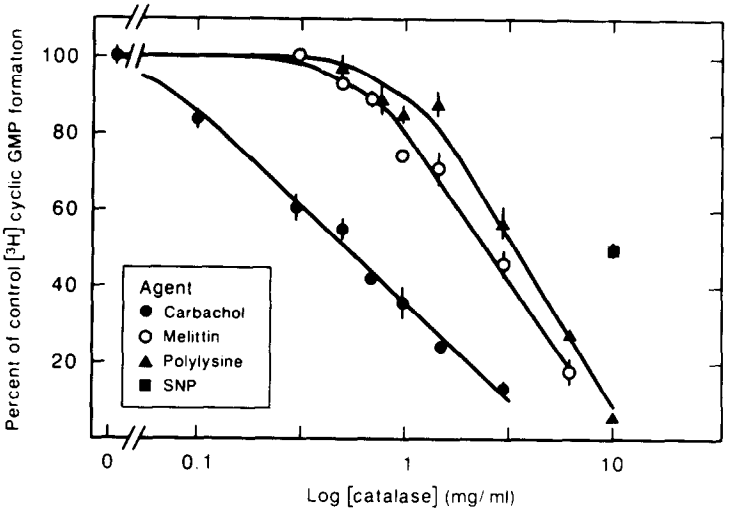

Fig. 5. Inhibition of carbachol (1 mM)-, melittin (10 μg/ml)-, polylysine (100 μg/ml)-, and sodium nitroprusside (SNP) (500 μM)-mediated cyclic GMP formation by catalase. Various concentrations of catalase (mg/ml) were incubated with N1E-115 cells at 37° for 30 min before the stimulants were added. Time of stimulation was 30 sec with carbachol and 10 min with the other agents. The experiment showing inhibition of the muscarinic receptor response is typical of at least four independent experiments; the other assays were performed once. These assays were performed in triplicate. The IC_{50} values were (vs stimulant): 2 μM (carbachol); 8 μM (melittin); 12 μM (polylysine); and 40 μM (sodium nitroprusside). The molecular weight of polylysine used was 40,000 daltons.

columns. This material was found to be composed of a mixture of at least seven products (data not shown). The profile of ultraviolet absorbance of this mixture gave an absorbance maximum of 235 nm, indicating the presence of conjugated double bonds resulting from probable hydroxylation of the arachidonate. Peak A inhibited the muscarinic receptor-mediated cyclic GMP response with an IC_{50} value of 4 μM (Table 1).

The finding that oxidized derivatives of arachidonic acid could block receptor-mediated cyclic GMP formation (Figs. 2 and 3) suggested that enzymatically-synthesized arachidonate metabolites would also inhibit this response. Two monoHETEs were manufactured, purified, and tested. 15-HETE, synthesized by soybean lipoxygenase, caused 50% inhibition at 8 μM and 12-HETE synthesized by the platelet lipoxygenase inhibited with an IC_{50} of 17 μM (Table 1). In a further experiment with prostaglandin $F_{2\alpha}$, this agent did not inhibit the cyclic GMP response even at concentrations exceeding 100 μM (data not shown).

Specificity of the arachidonic acid inhibitors. To determine whether other effector systems activated by muscarinic receptors in N1E-115 cells involved the metabolism of arachidonic acid, the effects of ETYA and NDGA on the muscarinic receptor-mediated inhibition of prostaglandin E_1-stimulated cyclic AMP were studied. ETYA and NDGA, at concentrations that would completely or substantially block cyclic GMP formation, had no effect on the degree to which carbachol (1 mM) inhibited the prostaglandin E_1 (1 μM)-mediated cyclic AMP response (Fig. 4). Similar results were obtained with 15-HETE (M. McKinney, manuscript submitted for publication). These findings gave further support for the separate identity of the muscarinic receptor–effector complexes involved in alterations in cyclic GMP and cyclic AMP levels.

In several other experiments, the effect of ETYA on the ability of the muscarinic receptor to stimulate the release of inositol phosphates from N1E-115 cells was evaluated (data for one experiment are given in Table 2). ETYA in this experiment did not inhibit, but actually slightly enhanced, the stimulated release of inositol phosphates. However, the effect of 50 μM ETYA on the receptor-stimulated release was quite variable; in two experiments it potentiated the response, while in two others it partially inhibited it. The average response in the presence of ETYA was 94 ± 29% of the control (N = 4). ETYA did not affect the basal response. When lithium ion (10 mM) was present, ETYA blocked the response completely (one experiment). In other experiments, 15-HETE did not inhibit the response (M. McKinney, manuscript submitted for publication).

Inhibition of muscarinic receptor-mediated cyclic GMP formation by catalase. Several studies of guanylate cyclase activation in the literature have indicated that this enzyme may be activated by lipid peroxides or by the hydroxyl radical. In several experiments, we studied the effects of various oxygen-derived radicals on the N1E-115 muscarinic receptor-mediated cyclic GMP response. A number of radical-generating systems were used (peroxidation of membrane lipids by xanthine oxidase/hypoxanthine/$FeCl_3$/ADP, the generation of oxygen radicals by auto-oxidation of 6-hydroxydopamine, or the generation of lipid peroxide with soybean lipoxygenase). In general, all these systems led to the rapid inhibition of the muscarinic receptor-mediated cyclic GMP response (data not shown). In the course of these studies, it was found that the hydrogen peroxide-scavenging enzyme catalase was an inhibitor of this response (Fig. 5 and Table 1). The IC_{50} for catalase in blocking the cyclic GMP response to 1 mM carbachol was about 5 μM. This inhibition was rapid, being complete within 2 min (data not shown). As this hemoprotein (molecular weight 250,000) would not be expected to cross the membrane into the neuroblastoma cell cytosol, its inhibition of the muscarinic receptor cyclic GMP response indicated that it was interacting with a receptor and/or effector system on the cell surface or that it was capable of degrading a lipid-soluble second messenger, which was formed in the cytosol and could freely diffuse out of the cell. This possibility was supported by the fact that catalase demonstrated lesser potency in blocking the cyclic GMP stimulation by two other agents which are more efficacious than the muscarinic receptor (melittin and polylysine, Fig. 5). Details of our studies of these two non-receptor agents will be reported elsewhere. Catalase was least effective in inhibiting the cyclic

Table 3. Effect of catalase on the muscarinic inhibition of prostaglandin E_1-mediated elevation of cyclic AMP levels in N1E-115 cells

Experiment	[^{3}H]Cyclic AMP formed over basal (dpm/million cells)	
Control		
Basal	10,800 ± 600	
PGE_1 (10 μM)	63,000 ± 2,000	
PGE_1 + Carbachol (1 mM)	39,600 ± 600	(−45%)
Catalase-treated		
Basal	10,500 ± 600	
PGE_1 (10 μM)	73,000 ± 1,000	
PGE_1 + Carbachol (1 mM)	39,000 ± 1,000	(−54%)

Catalase (12 μM) was incubated with N1E-115 cells for 30 min at 37° before the addition of the prostaglandin (10 μM) for 10 min, or the addition of the prostaglandin (10 μM) + carbachol (1 mM) for 10 min. This experiment was performed with sextuplets and is one of two.

GMP response to sodium nitroprusside, an agent which directly activates guanylate cyclase after diffusing into the interior of the cell (Fig. 5). Treatment of N1E-115 cells with catalase did not affect the muscarinic receptor-mediated reduction of PGE_1-mediated elevation of cyclic AMP levels (Table 3). The enzyme also did not affect the muscarinic receptor-mediated release of [^{3}H]inositol phosphates (Table 2) and, in this experiment, significantly potentiated the response. Catalase did not affect the basal release (in one experiment).

DISCUSSION

Several well-known lipoxygenase inhibitors were shown to inhibit receptor-mediated cyclic GMP formation in N1E-115 cells. Cyclooxygenase inhibitors were ineffective in blocking this response except at inordinately high concentrations. Agents that disturb the oxidation–reduction state of the cell were shown to interfere with the cyclic GMP response. Muscarinic receptor-mediated inhibition of the elevation of [^{3}H]cyclic AMP by prostaglandin E_1 was not affected by lipoxygenase inhibitors. The muscarinic receptor-induced release of inositol phosphates (without lithium ion present) was also insensitive to ETYA. Arachidonic acid and oxidative metabolites of it were inhibitory to the receptor-mediated increases in [^{3}H]cyclic GMP levels; 15-HETE was as potent as ETYA in the blockade of this response. Catalase was an effective inhibitor of the muscarinic receptor-mediated cyclic GMP response, whereas it had no inhibitory effect on the other two muscarinic responses assayed.

We have shown that, in an intact and functional neuroblastoma cell preparation, a particular response mediated by one muscarinic receptor subtype involves an oxidative process, which displays some pharmacological specificity. The data suggest that the receptor can activate a lipoxygenase-type reaction that leads to the activation of guanylate cyclase, as previously proposed [5]. The IC_{50} values found for ETYA, NDGA, and 15-HETE are sufficiently low to indicate possible action of these drugs at a lipoxygenase. However, the low potency of BW755c, AA861, FPL 55712, and FPL 57231 would probably rule out the existence of a 5-lipoxygenase. The preferential blockade by arachidonic acid metabolites or analogues suggested that it was a specific enzymatic process of arachidonic acid oxidation that was involved in cyclic GMP stimulation. Of particular interest was 15-HETE, as it has been shown to be a relatively potent inhibitor of lipoxygenase and membrane-linked processes in other cell types and a mediator of vasoconstriction in cerebral arteries. Further studies with 15-HETE are reported elsewhere ([17]; M. McKinney, manuscript submitted for publication).

In our experiments in which we have studied N1E-115 enzymatic synthesis of eicosanoids, we have obtained data indicating that these cells synthesize only low amounts of the prostaglandins, and little, if any, of the HETEs. Lipid hydroperoxides generated in the presence of an homogenate of N1E-115 cells have so far only led to the inhibition of guanylate cyclase. Thus, the point at which the inhibitors used in this study act to block the response to the receptor is not yet known. The finding that agents that generally perturb oxidation processes have effects on cyclic GMP formation would point toward some kind of electron transfer process involving oxygen. However, many of these agents can also affect the physical state of the membrane (e.g. α-tocopherol); it is not yet clear as to which aspects of the inhibitors are related to the effects on the receptor-activated mechanism.

Our data demonstrate that responses to neural muscarinic receptors can be selectively blocked by pharmacologic agents. These findings, besides having fundamental significance with regard to muscarinic receptor mechanisms, relate to the understanding of receptor function in pathologic brain mechanisms. Though the physiologic functions of cyclic nucleotides, particularly cyclic GMP, are incompletely understood at this time, brain mechanisms responsible for the modulation of their levels are known to exist. Protein kinases, which presumably act to phosphorylate specific cellular proteins in response to changes in cyclic nucleotide levels, have been shown to be present in the brain. Thus, it is likely to be of some importance to understand how the modulation of the levels of these second messengers relates to overall brain function. Normally the levels of unesterified fatty acids in the brain are kept at a low level as the acyl-CoA transferase continually re-esterifies the fatty acid into phospholipids. During ischemia or other pathologic states, the levels of the unesterified fatty acids rise; of particular concern are the unsaturated fatty acids, of which arachidonic acid composes a major part. Spontaneous peroxidation, either by chemical or enzymatic means, of released arachidonic acid can occur. Many metabolites of arachidonic acid are modulators of cellular function; the other products of oxidation might be expected to have deleterious effects. Such metabolites could affect neuronal receptor function, especially if the effector system involves lipid oxidation in generating second messengers.

Presently, it is not known how the various arachidonic acid metabolites or oxygen-derived radicals exert their inhibitory effects on the cyclic GMP response in N1E-115 cells. Oxygen-derived free radicals could directly oxidize receptors, their second messengers, or the guanylate cyclase itself, while fatty acids could change membrane permeability, interfere with cellular radical-generating mechanisms, or inhibit specific intracellular eyzymes. The HETEs may act to block the putative lipoxygenase involved in the cyclic GMP response to the receptor, or act at some other enzyme for which arachidonic acid is a substrate. A leukotriene produced by the lipoxygenase pathway does not appear to be the second messenger for guanylate cyclase stimulation as the leukotrienes B_4, C_4, D_4, and E_4 at concentrations up to 10 μM did not stimulate [^{3}H]cyclic GMP synthesis in a homogenate of N1E-115 cells (data not shown).

The result with catalase provides a clue as to the nature of this second messenger, as the hemoprotein was effective in low micromolar concentrations at blocking the muscarinic receptor-mediated cyclic

GMP response. Catalase was less effective against polylysine and melittin. In terms of the maximal cyclic GMP formed, the order of efficacy for these agents in mediating cyclic GMP formation is: polylysine > melittin > carbachol (M. McKinney and E. Richelson, unpublished information). If the efficacy for stimulating cyclic GMP formation is dependent upon the amount or rate of second messenger generation, this would explain the decreased inhibitory ability of catalase with regard to the two non-receptor agents.

Acknowledgements—Supported by NIH Grants NS21319 and MH27692, and by the Mayo Foundation. AA861 was a gift of Dr. R. Abraham. ETYA was a gift of Hoffmann-La Roche. BW755c was a gift of Burroughs-Welcome. FPL 57231 and FPL 55712 were gifts of Dr. P. Vanhoutte.

REFERENCES

1. M. McKinney and E. Richelson, *A. Rev. Pharmac. Toxic.* **24**, 121 (1984).
2. M. McKinney, S. Stenstrom and E. Richelson, *Molec. Pharmac.* **26**, 156 (1984).
3. M. McKinney, S. Stenstrom and E. Richelson, *Molec. Pharmac.* **27**, 223 (1985).
4. R. M. Snider and E. Richelson, *Science* **221**, 566 (1983).
5. R. M. Snider, M. McKinney, C. Forray and E. Richelson, *Proc. natn. Acad. Sci. U.S.A.* **81**, 3905 (1984).
6. D. Leiber and S. Harbon, *Molec. Pharmac.* **21**, 654 (1981).
7. N. D. Goldberg, G. Graff, M. K. Haddox, J. H. Stephenson, D. B. Glass and M. E. Moser, *Adv. Cyclic. Nucleotide Res.* **9**, 101 (1978).
8. C. K. Mittal and F. Murad, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4360 (1977).
9. E. Richelson, F. G. Prendergast and S. Divinetz-Romero, *Biochem. Pharmac.* **27**, 2039 (1978).
10. S. Stenstrom and E. Richelson, *J. Pharmac. exp. Ther.* **221**, 334 (1982).
11. J. M. Boeynaems, A. R. Brash, J. A. Oates and W. C. Hubbard, *Analyt. Biochem.* **104**, 239 (1980).
12. G. Graff, *Meth. Enzym.* **86**, 386 (1982).
13. C. P. A. Van Os, G. P. M. Rijke-Schilder, H. Van Halbeek, J. Verhagen and J. F. G. Vliegenthart, *Biochim. biophys. Acta* **663**, 177 (1981).
14. S. Shak, H. D. Perez and I. M. Goldstein, *J. biol. Chem.* **258**, 14948 (1983).
15. F. F. Sun, *Meth. Enzym.* **72**, 435 (1981).
16. M. Dixon, *Biochem. J.* **55**, 170 (1953).
17. M. McKinney, *Soc. Neurosci. Abstr.*, **11**, 655 (1985).