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The Putative M_1 Muscarinic Receptor Does Not Regulate Phosphoinositide Hydrolysis

Studies with Pirenzepine and McN-A343 in Chick Heart and Astrocytoma Cells

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SUMMARY

Muscarinic receptor activation stimulates phosphoinositide hydrolysis and inhibits cyclic AMP formation in dissociated embryonic chick heart cells. We used this preparation to examine the hypothesis that the putative M_1 and M_2 receptor subtypes are selectively coupled to these two responses. Atropine blocks the effects of carbachol on cyclic AMP formation and phosphoinositide breakdown with nearly identical K_I values (1.9 and 0.8 nM); these values are close to the apparent K_D (1.8 nM) of atropine competition for [^{3}H] *N*-methylscopolamine binding. Pirenzepine blocks the effect of carbachol on cyclic AMP formation with a K_I of 48 nM, a value similar to the apparent K_D (23 nM) determined in radioligand-binding studies. In contrast, a higher concentration of pirenzepine is needed to inhibit carbachol-stimulated phosphoinositide hydrolysis (K_I = 255 nM). Two selective agonists, McN-A343 and AHR 602, inhibit cyclic AMP formation but do not stimulate phosphoinositide hydrolysis in chick heart cells. Muscarinic receptor-mediated phosphoinositide hydrolysis in 1321N1 astrocytoma cells is also insensitive to McN-A343 or AHR 602 and is antagonized only by relatively high concentrations of pirenzepine. The M_1 receptor, as previously defined, has high affinity for pirenzepine and is activated by McN-A343. We find that these ligands have greater activity at muscarinic receptors that inhibit cyclic AMP formation than at those that stimulate phosphoinositide hydrolysis. Thus, if different receptor subtypes are associated with these two responses, the M_1 receptor regulates cyclic AMP rather than phosphoinositide metabolism. Our data also demonstrate that the chick heart has muscarinic receptors with high affinity for pirenzepine, and thus, in contrast to rat heart, appears to have predominantly M_1 receptors.

INTRODUCTION

Muscarinic cholinergic receptors are present throughout the body and mediate a variety of physiological and biochemical responses. There are a number of subtle differences in the pharmacological properties of the receptors that mediate these diverse physiological responses (1–6). For example, differences in muscarinic receptors are revealed both with partial agonists (2, 4, 5) and with antagonists that act at allosteric sites (3, 6–8). These findings suggest, but do not prove, that there are subtypes of muscarinic receptors, and they have led to a search for muscarinic receptor antagonists with tissue selectivity. Using radioligand-binding studies, Hammer *et al.* (9) first demonstrated that the muscarinic antagonist pirenzepine exhibits up to 50-fold higher apparent affinity for muscarinic receptors in certain brain regions than for those in heart or smooth muscle. This, and the finding that [^{3}H]pirenzepine labels a subclass of receptors that predominate in sympathetic ganglia and certain brain regions (10–12), has led to the hypothesis that there are subtypes of muscarinic receptors that are distinguished by pirenzepine.

Physiological studies have looked for functional correlates of the high and low affinity pirenzepine-binding sites. Pirenzepine does not have selectivity for blocking pre- versus postsynaptic muscarinic receptors (13), but does show selectivity in blocking vagal effects on gastric secretion versus heart rate (14) and for blocking agonist-induced depolarization of the ganglion versus contraction of the ileum (6). Certain agonists such as McN-A343 and AHR 602 also appear to be selective because they show greater stimulatory activity (i.e., appear more efficacious) at muscarinic receptors in sympathetic ganglia and brain than at those in the ileum or heart (5, 15–17).

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On the basis of these observations, muscarinic receptors have been tentatively subclassified. The term M_1 has been used to describe the muscarinic receptor in brain and ganglion that has high affinity for pirenzepine and is activated by McN-A343 and AHR 602 (11, 16–18). The term M_2 has been applied to the receptor with low affinity for pirenzepine that appears to predominate in heart and ileum (11, 17, 18). The M_1 receptor has been suggested to be independent of a guanine nucleotide regulatory protein because GTP does not regulate [^{3}H] pirenzepine binding (11, 12). The M_2 receptor has been suggested to regulate adenylate cyclase because this receptor subtype predominates in rat heart (9, 10), a tissue in which muscarinic receptors are known to couple to inhibition of adenylate cyclase (19). These findings have led to speculation that the M_1 receptor mediates phosphoinositide hydrolysis and calcium mobilization whereas the M_2 receptor mediates adenylate cyclase inhibition (10).

To test directly the proposed relationship of M_1 and M_2 receptors to phosphoinositide hydrolysis and adenylate cyclase inhibition, one needs to study the two responses in a single system and under similar conditions. We recently showed that muscarinic receptors regulate both of these responses in cardiac preparations including embryonic chick heart cells (20, 21). The studies presented here compare the effects of the M_1-specific agonists and antagonists on the muscarinic receptors that inhibit cyclic AMP accumulation and those that stimulate phosphoinositide hydrolysis. We show here that, contrary to what has been suggested, pirenzepine and McN-A343 are *less* effective at receptors regulating phosphoinositide turnover than at those regulating cyclic AMP metabolism. Additionally, we show that muscarinic receptors in the chick heart have relatively high affinity for pirenzepine, and by this criterion, the predominant receptor in the chick heart is of the M_1 rather than M_2 receptor subtype.

Because our observations on the chick heart cells were not consistent with previously postulated relationships, we expanded our study to include the 1321N1 astrocytoma cell. The data obtained using this cultured cell line further support our conclusion that the receptor associated with phosphoinositide hydrolysis cannot be characterized as an M_1 receptor.

EXPERIMENTAL PROCEDURES

Cell preparations. Heart cells were prepared from 13-day-old chick embryo hearts, as described previously (21). Briefly, the cells were enzymatically dispersed in Ca^{2+}-Mg^{2+}-free buffer containing 0.25% trypsin. Heart cells were separated from erythrocytes and cell debris by centrifugation in 30% Percoll for 30 min at 20,000 × *g*. The final cell preparation was 80–90% viable (by trypan blue exclusion) and the yield of cells was 5–10 × 10^6 cells/heart. Dissociated cells were assayed within 1 hr of preparation.

1321N1 astrocytoma cells were cultured as described previously (22, 23) in Dulbecco's modified Eagle's medium with 5% fetal calf serum. All assays were performed with confluent 7-day-old cultures grown on 35-mm plates.

Assay conditions. All assays were carried out at 35–37° in PSS[3] buffered to pH 7.4 with either 20 mM HEPES (for 1321N1 cells and for all radioligand-binding assays) or with 25 mM $NaHCO_3$ (chick heart cells). The standard PSS buffer was comprised of 118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose; for astrocytoma cells, the buffer contained 3 mM $CaCl_2$ and 0.5 mM Na_2EDTA. The heart cells were assayed in suspension and were gassed with 95% O_2–5% CO_2 throughout the assay.

Cyclic AMP assays. Chick heart cells (~7 × 10^6 cells/ml) were equilibrated with 100 μM isobutylmethylxanthine and the desired antagonist for 20 min prior to addition of ascorbate (vehicle), isoproterenol, or isoproterenol plus carbachol for 2 min. To end the assay, cells were centrifuged for 30 sec at full speed in a Beckman Microfuge B, medium was replaced with 10% trichloracetic acid, and the cells were sonicated. Cyclic AMP was purified and assayed by the competitive protein-binding assay of Gilman, as previously described (24). Protein was assayed by the method of Bradford (25).

Phosphoinositide assays. Phosphoinositide hydrolysis was monitored as previously described (20) by measuring [^{3}H]Ins1P accumulation in the presence of LiCl, an inhibitor of the enzyme that converts Ins1P to inositol (26). For assays with chick heart cells, phosphoinositides were radiolabeled by incubating the cells for 1 hr in PSS containing 5–10 μCi/ml myo[2-^{3}H]inositol. Cells were assayed at a concentration of approximately 10^7 cells/ml. Assays were initiated by the addition of LiCl (10 mM) and agonists (antagonists were added 20 min earlier). The assay, which is linear for at least 60 min, was routinely terminated 30 min after agonist addition (some experiments were terminated at 5 min) by centrifugation of the cells and sonication in chloroform/methanol (1:2). Additional chloroform and water were added and the resulting two-phase solution was clarified by centrifugation. [^{3}H]Inositol 1-phosphate was separated from [^{3}H]inositol by anion exchange chromatography with a modification (20) of the procedure described by Berridge *et al.* (26).

In assays with 1321N1 cells, phosphoinositides were radiolabeled by incubating the monolayers of confluent 35-mm plates overnight in normal growth medium containing 1 μCi/ml myo[2-^{3}H]inositol. At the time of the assay, monolayers were washed with PSS (37°) and incubated with antagonists for 10 min. LiCl and agonists were added for an additional 10 min. Assays were terminated by removing the drug solution by aspiration and adding cold methanol. Cells were removed from the plate by scraping and were sonicated in chloroform/methanol/water (5:10:4). [^{3}H]Inositol 1-phosphate was quantitated as described for the chick heart cells.

Radioligand-binding assays. Binding assays on intact chick heart cells were carried out using cells suspended in PSS, containing either [^{3}H]QNB (0.1 nM) or [^{3}H]NMS (0.4–1.0 nM). Incubations were carried out for 60–75 min at 35° and were terminated by lysing the cells with hypotonic buffer and filtering. Nonspecific binding (that not inhibited by 10 μM atropine) was <15% for [^{3}H]QNB and <25% for [^{3}H]NMS at these concentrations, which are three to six times their respective K_D (15 pM for QNB, 165 pM for NMS). Both ligands bound to an apparently homogeneous population of binding sites. The total number of binding sites for the impermeant ligand [^{3}H]NMS in intact heart cells was approximately half of that for [^{3}H]QNB. The differential binding capacity for these two ligands has been noted by other investigators[4] and previously described in 1321N1 cells (29, 36). The same K_D values were calculated for atropine or for pirenzepine using either radioligand. Competition curves were analyzed by means of a weighted nonlinear least squares computer curve-fitting program, LIGAND, as described previously (8).

[3] The abbreviations used are: PSS, physiological salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMS, *N*-methylscopolamine; QNB, quinuclidinylbenzilate; Ins1P, inositol 1-phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[4] A. Sastre, personal communication; E. El Fakahany, personal communication.

Radioligand-binding studies on intact 1321N1 cells were carried out with cells in monolayer. Monolayers were washed three times with 1.5 ml of PSS to remove the growth medium. The cells were then incubated for 75–90 min with 2.5 ml of PSS containing 0.5 nM [^{3}H]NMS. Incubations were terminated by washing the monolayers four times with 3 ml of PSS. Cells were solubilized in 0.5 ml of a solution containing 5 mM EGTA, 1 mM HEPES, and 3% (w/v) Triton X-100, removed from the plate by scraping, and counted. Nonspecific binding (that not inhibited by 10 μM atropine) was <20% of the total [^{3}H]NMS binding. Saturation binding isotherms with [^{3}H]NMS were consistent with a single population of radioligand-binding sites (~6000 sites/cell) with a K_D of 60 pM.

Materials. Fertilized White Leghorn chicken eggs were obtained from McIntyre Poultry and Eggs (San Diego, CA). Fetal calf serum and Dulbecco's modified Eagle's medium were from Irvine Scientific. Atropine sulfate and carbamylcholine chloride (carbachol) were from Sigma Chemical Company. Pirenzepine was a gift from Dr. Barry Wolfe, University of Pennsylvania and Dr. Donald Jenden, University of California, Los Angeles. McN-A343 was from McNeil. AHR 602 was from A. H. Robins. Oxotremorine-M was a gift from Drs. Bjorn Ringdahl and Donald Jenden, U. C. L. A. Myo[2-^{3}H]inositol, [^{3}H]QNB, and [^{3}H]NMS were from New England Nuclear.

RESULTS

We have previously shown that the cholinergic agonist carbachol inhibits isoproterenol-stimulated cyclic AMP accumulation and stimulates phosphoinositide hydrolysis in dissociated embryonic chick heart cells (21). These two responses exhibit quite different sensitivity to carbachol: the K_{act} for inhibition of cyclic AMP accumulation is 0.2 μM, whereas that for stimulation of Ins1P formation is 20 μM. The possible basis for this difference was the subject of our earlier paper (21).

In the experiments shown in Fig. 1, carbachol is used at five times the K_{act} for the appropriate response (1 μM for cyclic AMP inhibition, 100 μM for phosphoinositide hydrolysis). The nonselective muscarinic antagonist atropine blocks the ability of carbachol both to inhibit cyclic AMP accumulation and to stimulate Ins1P formation (Fig. 1) at nearly the same concentration. Similar K_I values are calculated for antagonism of the cyclic AMP and phosphoinositide response by atropine (1.9 and 0.8 nM, respectively; Table 1). The apparent K_D for atropine was also determined in radioligand-binding studies using either [^{3}H]QNB (Fig. 2) or [^{3}H]NMS (data not shown) as radioligand. The apparent K_D is the same with either radioligand and averages 1.8 nM (Table 1). Thus, atropine inhibits radioligand binding and muscarinic receptor-mediated changes in phosphoinositide and cyclic AMP metabolism with nearly the same apparent affinity.

The selective muscarinic antagonist pirenzepine is less potent than atropine for antagonizing either functional response, especially carbachol-stimulated phosphoinositide hydrolysis (Fig. 1). The K_I calculated for pirenzepine blockade of Ins1P formation is 255 nM, whereas the K_I for antagonism of the cyclic AMP response is 48 nM (Table 1). Radioligand binding to intact chick heart cells with either [^{3}H]QNB (Fig. 2) or [^{3}H]NMS (data not shown) gives an average K_D for pirenzepine of 23 nM. The average Hill slope of the pirenzepine competition

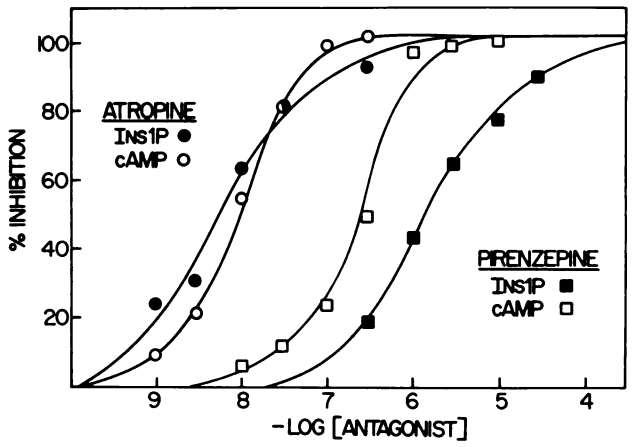

FIG. 1. *Antagonism of muscarinic receptor-mediated cyclic AMP and Ins1P responses by atropine and pirenzepine in chick heart cells*

Data are expressed as per cent inhibition of carbachol-stimulated [^{3}H]Ins1P accumulation or of carbachol-inhibited (isoproterenol-stimulated) cyclic AMP accumulation. Absolute values for cyclic AMP (picomoles/mg of protein) were: basal, 12; isoproterenol, 43; isoproterenol plus carbachol, 22. For Ins1P formation, absolute values were: basal, 153 cpm; carbachol, 486 cpm. Values are means from two separate experiments in each of which three to five separate determinations were made at each antagonist concentration.

TABLE 1

Comparison of apparent K_D and K_I values for atropine and pirenzepine effects on [^{3}H]QNB binding, cycle AMP accumulation, and phosphoinositide hydrolysis in chick heart cells

The apparent K_D was derived from the IC_{50} determined in competition binding experiments by the method of Cheng and Prusoff (35). Values shown are the geometric means ± standard error from three to four radioligand-binding experiments (like that shown in Fig. 2). The K_I values were also derived from IC_{50} values. The K_I values were determined separately (values given in parentheses) for each of the two experiments that were combined graphically in Fig. 1.

	K_D binding	K_I	
		Cyclic AMP	Ins1P
		nM	
Atropine	1.8 ± 0.5	1.9 (1.2; 3.1)	0.8 (0.6; 1.0)
Pirenzepine	23 ± 1.0	48 (45; 50)	255 (250; 260)

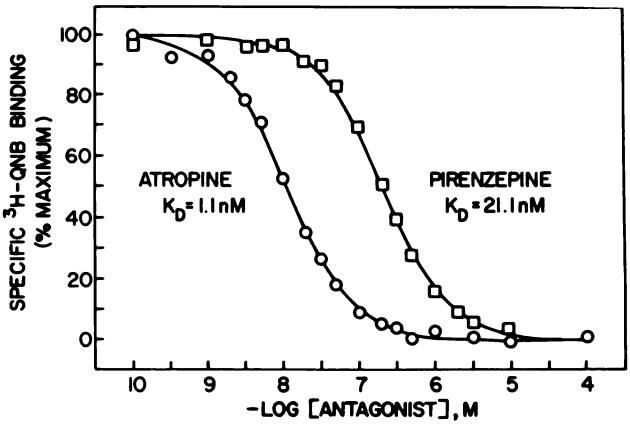

FIG. 2. *Competition for [^{3}H]QNB binding to chick heart cells by atropine and pirenzepine*

Values shown are averages of triplicate determinations from a representative experiment. The K_D was calculated from the IC_{50} as described by Cheng and Prusoff (35). The [^{3}H]QNB concentration was 0.11 nM.

curves on chick heart cells is 1.08, indicating a homogeneous population of binding sites. This apparent K_D for pirenzepine binding is similar to the K_I determined for functional antagonism of the cyclic AMP response. In contrast, the K_I for antagonism of Ins1P formation by pirenzepine (255 nM) does not agree with either of these values.

To confirm the K_I values obtained for pirenzepine in the experiments described above, these values were independently determined by Schild analysis (27). The concentration response relationships for stimulation of Ins1P accumulation by carbachol in the absence and the presence of various concentrations of pirenzepine are shown in Fig. 3. Increasing concentrations of pirenzepine cause parallel shifts in the concentration response curves for carbachol. This is characteristic of a competitive antagonist. The concentration response curves for carbachol-induced InsP formation are somewhat shallow (Hill slopes of ~0.8). The reason for this is not yet clear, although this was also noted for a brain synaptosomal preparation (28) and we see the same phenomenon in 1321N1 astrocytoma cells. Schild analysis of the data shown in Fig. 3 yields a K_I for pirenzepine of 240 nM, a value almost identical to that determined from the data in Fig. 1 (255 nM). Similar experiments were performed to determine the K_I for antagonism of the cyclic AMP response by pirenzepine (Fig. 4). These experiments yielded a K_I of 60 nM, also similar to the K_I calculated in experiments like those shown in Fig. 1. These data further demonstrate the disparity in the K_I of pirenzepine for antagonizing the two functional responses.

In the studies described above, the phosphoinositide response was measured in a 30-min assay. This time was used because carbachol-stimulated InsP formation is linear for at least 90 min (29), and the signal at much shorter times is very small. The cyclic AMP response was measured in a 2-min assay because desensitization appears to occur at longer times. One may consider, however, that the differential effect of pirenzepine is in some way related to the differences in assay time. We therefore carried out additional experiments in which both responses were assayed at 5 min and the K_I for pirenzepine was determined (data not shown). The values were remarkably similar to those given above: 230 nM for blockade of InsP formation and 67 nM for blockade of cyclase inhibition by pirenzepine.

Two agonists thought to be selective for M_1 receptors, McN-A343 and AHR 602, were compared for their ability to inhibit cyclic AMP formation and to stimulate phosphoinositide hydrolysis in chick heart cells. These agonists were tested at 1 mM and were compared to carbachol and to oxotremorine-M, a quaternary analog of oxotremorine which (unlike oxotremorine) acts as a full agonist (30). McN-A343 and AHR 602 cause 56 and 44%, respectively, of the maximal change in cyclic AMP accumulation caused by carbachol (Fig. 5). However, these two agents cause virtually no increase in Ins1P accumulation (Fig. 5).

The high affinity (23 nM) of the chick heart muscarinic receptor for pirenzepine was unexpected because heart has been categorized as a tissue in which most muscarinic receptors have low affinity for pirenzepine. To study this apparent discrepancy, we carried out several additional experiments. First, since previous analyses used membrane preparations, whereas our studies were with intact cells, we carried out pirenzepine competition experiments on membranes from 13-day embryonic chick heart. The K_D for pirenzepine determined in two separate

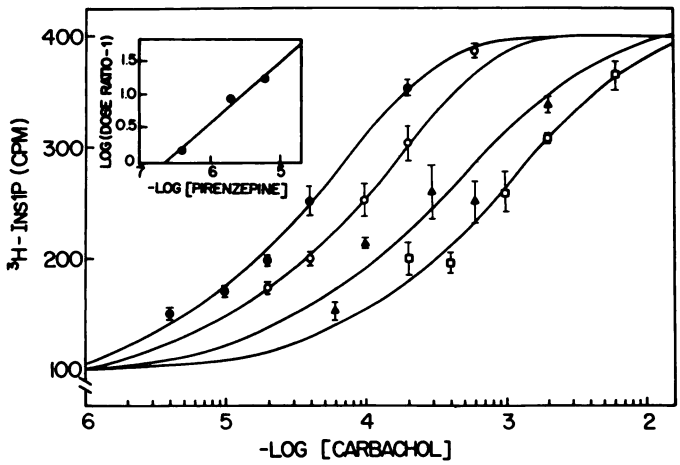

FIG. 3. *Schild analysis of antagonism of carbachol-stimulated [^{3}H] Ins1P formation by pirenzepine*

Concentration response relationships for stimulation of Ins1P accumulation in heart cells were determined in the absence of pirenzepine (●), or at pirenzepine concentrations of 0.4 (○), 2 (▲), and 6 μM (□). The log of the dose ratio (the ratio of the concentration of carbachol that causes a half-maximal response in the presence of pirenzepine to the concentration of carbachol that causes a half-maximal response in the absence of pirenzepine) minus 1 is plotted as a function of the log of the pirenzepine concentration in the inset. Linear regression of the dose ratio values yields a slope of 0.97. The K_D value as determined from the antilog of the extrapolated x intercept is 240 nM.

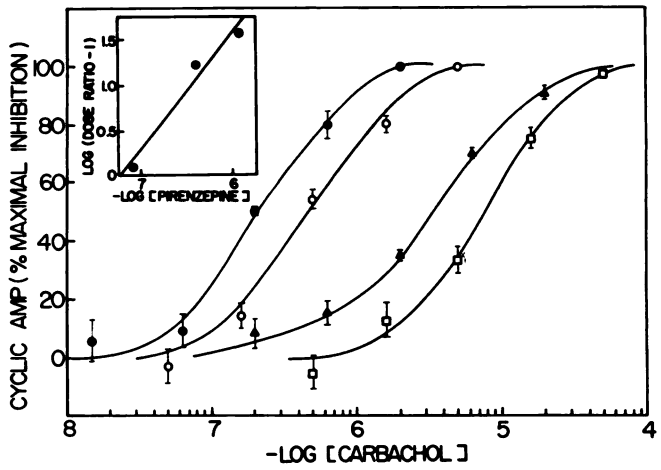

FIG. 4. *Schild analysis of antagonism of carbachol inhibition of cyclic AMP accumulation by pirenzepine*

Concentration response relationships for inhibition of isoproterenol-stimulated cyclic AMP formation in chick heart cells were determined in the absence of pirenzepine (●) or at concentrations of 0.08 (○), 0.4 (▲), and 1.2 μM (□). The inset is as described in the legend to Fig. 4; the slope was 1.2 and K_D was 60 nM. If the slope was constrained to 1, the K_D was 37 nM. The absolute values for cyclic AMP (picomoles/mg of protein) were: basal, 19; isoproterenol, 121; isoproterenol plus carbachol, 38.

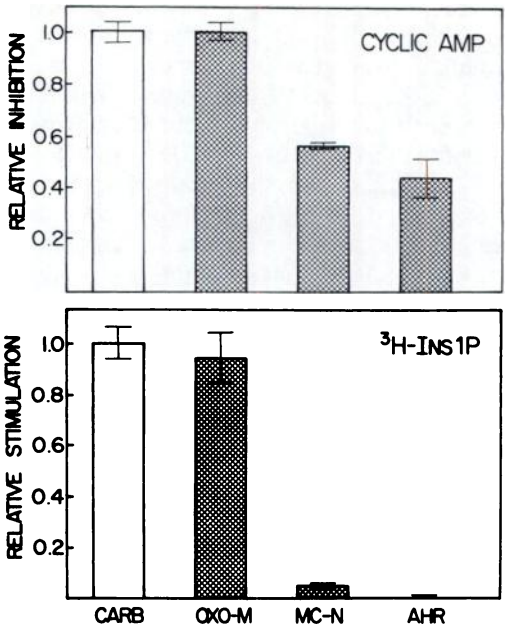

FIG. 5. *A comparison of the effects of McN-A343 and AHR 602 on cyclic AMP and Ins1P accumulation in chick heart cells*

The inhibition of isoproterenol-stimulated cyclic AMP accumulation (upper panel) and the stimulation of Ins1P accumulation (lower panel) by 1 mM AHR 602 (*AHR*), 1 mM McN-A343 (*MC-N*), and 100 μM oxotremorine-M (*OXO-M*; the quaternary derivative of oxotremorine) are expressed as a fraction of that caused by 100 μM carbachol (*CARB*). The absolute values for InsP formation were: control, 170 cpm; carbachol, 707 cpm. For cyclic AMP formation the absolute values (picomoles/mg of protein) were: basal, 12; isoproterenol, 59; isoproterenol plus carbachol, 22. Bars represent means ± standard error for six (cyclic AMP) or three (Ins1P) samples.

TABLE 2

Comparison of the apparent K_D for pirenzepine in membranes from chick hearts at different developmental stages and membranes from rat heart

The K_D is the apparent K_D value calculated from the IC_{50} obtained in competition experiments. The radioligand used was [^{3}H]QNB. Binding assays on rat heart membranes were carried out as described previously (8), in a PSS buffer like that used for chick heart membrane experiments.

	K_D pirenzepine
	nM
Chick heart membranes	
7-day embryonic	34
13-day embryonic	43
20-day embryonic	39
8-day posthatch	35
Rat heart membranes	800

experiments was 43 and 48 nM in the membrane preparation. Second, to determine if there might be developmental differences in pirenzepine binding, we examined membranes from hearts of chicks at different embryonic stages and from 8-day posthatch chicks; relatively high affinity pirenzepine-binding sites were present at every stage (Table 2). Third, we assayed membranes from rat heart. The K_D for pirenzepine was 800 nM, a 20-fold lower apparent affinity than that determined in parallel studies on the chick heart.

In 1321N1 astrocytoma cells, muscarinic receptors regulate calcium efflux and phosphoinositide metabolism (23, 29) but not adenylate cyclase activity (22). Both atropine and pirenzepine inhibit the phosphoinositide response to carbachol in 1321N1 cells (Fig. 6). Atropine blocks the stimulation of Ins1P formation with a K_I of 3 nM. Pirenzepine inhibits the stimulation of Ins1P formation in 1321N1 cells with a K_I of 170 nM. These values are similar to those obtained in our experiments on phosphoinositide hydrolysis in chick heart cells. Radioligand-binding studies give a K_D for atropine of 0.8 nM, and a K_D for pirenzepine of 113 nM (Fig. 7). The M_1-selective agonists shown to be poor activators of phosphoinositide hydrolysis in the chick heart cells are also ineffective at stimulating phosphoinositide hydrolysis in 1321N1 cells (Table 3).

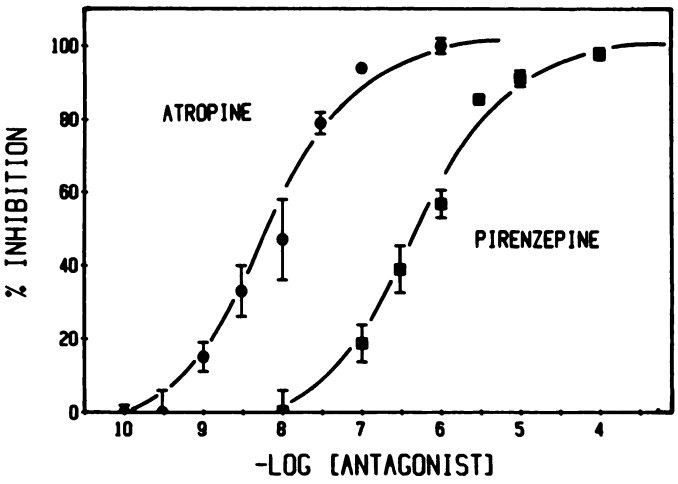

FIG. 6. *Inhibition of muscarinic receptor-mediated Ins1P accumulation by atropine and pirenzepine in 1321N1 cells*

Phosphoinositide hydrolysis was stimulated with 100 μM carbachol, a concentration 2.5 times the K_{act} for stimulation of InsP formation in these cells. Data shown are the per cent inhibition of [^{3}H]Ins1P accumulation, at the appropriate concentration of antagonist. Values are means ± standard error of four samples.

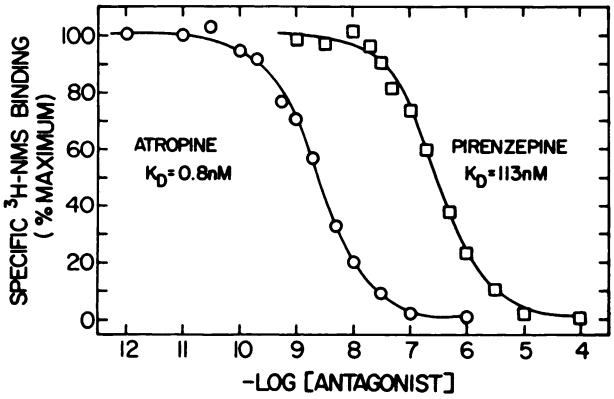

FIG. 7. *Competition of [^{3}H]NMS binding to intact 1321N1 cells by atropine and pirenzepine*

Values shown are averages of duplicate determinations from a representative experiment. The K_D was calculated from the IC_{50} (35). The concentration of [^{3}H]NMS used was 0.15 nM.

TABLE 3

Comparison of the effects of McN-A343 and AHR 602 on [^{3}H]Ins1P accumulation in 1321N1 cells

The concentration of all of the agonists was 100 μM. The [^{3}H]Ins1P values are means ± standard error for four to five samples.

Agonist	[^{3}H]Ins1P	Response
	cpm × 10^{-3}	%
Control	0.53 ± 0.02	
Carbachol	2.71 ± 0.11	100
McN-A343	0.58 ± 0.05	2
AHR 602	0.50 ± 0.03	0

DISCUSSION

The M_1 and M_2 muscarinic receptor subtypes were initially defined based on the effects of McN-A343 (16), and more recently on the basis of their affinity for pirenzepine. The affinity of the M_1 receptor for pirenzepine given in the literature ranges from approximately 5 to 30 nM (11, 12, 17) while the lower affinity (M_2) binding sites have K_D values for pirenzepine ranging from 200 to 800 nM (9, 17). It has been postulated that the M_1 receptor couples to and stimulates phosphoinositide hydrolysis and calcium mobilization while the M_2 receptor couples to and inhibits adenylate cyclase (10).

To determine the relationship between the putative muscarinic receptor subtypes and these functional responses, we examined the potency of pirenzepine for antagonizing cyclic AMP and phosphoinositide responses to carbachol and for competing for muscarinic receptor-binding sites. All of these responses were measured in the same system (the intact chick heart cell) and under nearly identical conditions. The results from studies with chick heart cells were corroborated by studies in a second preparation, the 1321N1 astrocytoma cell. The nonselective antagonist atropine was used as a standard for comparing the effects of pirenzepine on receptor binding, phosphoinositide hydrolysis, and cyclic AMP accumulation, and for comparing the two preparations.

The data presented here demonstrate that the muscarinic receptor that regulates phosphoinositide hydrolysis in chick heart cells and 1321N1 cells has relatively low affinity for pirenzepine. The K_I for antagonism of carbachol-stimulated Ins1P formation by pirenzepine is 240–255 nM in chick heart cells, and is 170 nM in 1321N1 cells. These values are not characteristic of a receptor with high affinity for pirenzepine; therefore, on this basis, the muscarinic receptor that mediates phosphoinositide hydrolysis in the chick heart cell and astrocytoma cell cannot be characterized as an M_1 receptor.

The agonists McN-A343 and AHR 602 have greater efficacy for stimulating physiological responses in ganglion than in heart or smooth muscle (5, 16, 17), and one of these (McN-A343) was used to define the M_1 muscarinic receptor. The observation that these two compounds cause virtually no increase in [^{3}H]Ins1P accumulation supports our conclusion that the receptor coupled to phosphoinositide hydrolysis in chick heart cells and 1321N1 astrocytoma cells cannot be classified as an M_1 receptor.

A separate question is whether the putatively M_1-selective agonists and pirenzepine distinguish between the receptors coupled to phosphoinositide and cyclic AMP metabolism. The M_1-specific agonists clearly can discriminate between the receptors regulating these two functional responses because they have virtually no effect on phosphoinositide hydrolysis, but they are reasonably efficacious for inhibiting cyclic AMP formation. Two full agonists, carbachol and the quaternary oxotremorine analog, oxotremorine-M, do not show such selectivity. Pirenzepine may also discriminate between receptors that couple to phosphoinositide and cyclic AMP metabolism in the chick heart, because pirenzepine blocks muscarinic receptor-mediated phosphatidylinositol hydrolysis with ~5-fold lower apparent affinity than that calculated for blockade of the cyclic AMP response. In contrast, the nonselective antagonist atropine blocks both functional responses with nearly the same K_I. These data demonstrate that pirenzepine and M_1-specific agonists do show selectivity compared to more classical agonists and antagonists. The 5-fold selectivity of pirenzepine is, however, far less than that used to define M_1 and M_2 receptor subtypes. Additionally, if the M_1 and M_2 classification is applied, it would be necessary to argue, counter to previous hypotheses, that cyclic AMP formation is regulated through an M_1 receptor, while phosphoinositide hydrolysis is regulated through a receptor with characteristics of an M_2 receptor.

The hypothesis that the receptors coupled to phosphoinositide and cyclic AMP responses differ in their affinity for pirenzepine is at odds with our observation that pirenzepine appears to bind to a single class of receptors in chick heart cells. If there is a subtype of muscarinic receptor that is associated with phosphoinositide hydrolysis in this preparation, and if this receptor has relatively low affinity for pirenzepine, one might expect to see evidence of heterogeneous binding in studies of pirenzepine competition for radioligand-binding sites. An explanation for the homogeneous binding of pirenzepine could be that the chick heart contains only a small proportion of low affinity receptors. Since the receptors regulating the two responses appear to vary only 5-fold in their affinities for pirenzepine, a subpopulation constituting less than 20% of the total receptors could go undetected in computer analysis of competition for radioligand binding (31). This would explain why we have been unable to fit our competition binding curves to a two-site model.

As discussed above, our data demonstrate that the M_1-selective agonists and pirenzepine recognize differences that are not discriminated by carbachol or atropine. The question we would raise is whether the differences seen with these ligands reflect heterogeneity in the receptors that couple to these two responses. The finding that McN-A343 and AHR 602 show selectivity, whereas the more efficacious agonists carbachol and oxotremorine-M do not, parallels our earlier observation on the differential effects of oxotremorine and carbachol on cyclic AMP and phosphoinositide metabolism (21). Agonist selectivity does not necessarily prove that different receptor subtypes mediate the two responses because it could also result from differences in the receptor reserve for the two responses. Relatively fewer agonist-occupied

receptors may be needed to fully inhibit adenylate cyclase than to stimulate phosphoinositide hydrolysis. The effects of agonists like McN-A343, which have low intrinsic activity, would be most affected by such differences.

The differences seen for pirenzepine are more likely to reflect differences in the receptors coupled to the two responses. One problem with this conclusion is that the nature of the coupling does not have a predictable effect on pirenzepine affinity. For example, Fisher and Bostus (32) report that in the striatum pirenzepine blocks phosphoinositide hydrolysis with a low affinity, equal to that which we see in chick heart, but this antagonist blocks phosphoinositide hydrolysis in hippocampus and cortex with relatively high affinity (32). Furthermore, Gil and Wolfe (33) find that in rat cortex pirenzepine blocks phosphoinositide hydrolysis with relatively high affinity and adenylate cyclase inhibition with relatively low affinity. Thus, the apparent effects of coupling on pirenzepine affinity in rat cortex are the opposite of those which we have described for chick heart cells.

The fact that there are differences in the K_I for pirenzepine antagonism of phosphoinositide metabolism in various tissues suggests that receptor classification, as defined by pirenzepine, does not correlate in any simple way with the functional properties of the muscarinic receptor. Additionally, the tissue selectivity of this antagonist is less simple than that initially proposed, since muscarinic receptors in the chick heart have relatively high affinity for pirenzepine, while those in the rat heart have low affinity. Thus, while it is clear that pirenzepine and McN-A343 show selectivity not manifested by other muscarinic agonists or antagonists, this selectivity may result from differences in the conformation, membrane environment, or allosteric sites associated with the receptor (15, 34) rather than from heterogeneity related to the association of the receptor with a specific effector.

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