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Differences in Muscarinic Receptor Reserve for Inhibition of Adenylate Cyclase and Stimulation of Phosphoinositide Hydrolysis in Chick Heart Cells

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SUMMARY

Carbachol is 100 times more potent for inhibiting cyclic AMP formation than for stimulating phosphoinositide (PI) hydrolysis in chick heart cells. To determine whether this reflects differences in agonist affinity of the receptor(s) coupled to the two responses, we measured these functional responses following removal of receptor reserve with propylbenzilylcholine mustard (PrBCM). Conditions of PrBCM treatment that led to progressive loss of up to 95% of the [^{3}H]-*N*-methylscopolamine-binding sites decreased the potency but not the maximal capacity of carbachol to inhibit cyclic AMP formation. In contrast, there was a marked decrease in the maximal PI response to carbachol. The K_A for carbachol, calculated by measuring functional responses following receptor inactivation, was similar whether the cyclic AMP or the PI response was examined. These K_A values (~40 μM) were similar to the K_D calculated by examining carbachol competition for [^{3}H]-*N*-methylscopolamine-binding sites on the intact cell. PrBCM treatment also decreased the maximal effect of oxotremorine on cyclic AMP formation under conditions in which carbachol remained a full agonist for this response. We interpret our data as indicating that: 1) there is much greater receptor reserve in the coupling of muscarinic receptors to adenylate cyclase than to PI hydrolysis; 2) this, rather than differences in receptor affinity underlies the disparate dose-response relationships for the two responses; and 3) differences in the effects of weak agonist on the two responses may also reflect differences in receptor reserve. We suggest that muscarinic receptors with the same affinity for carbachol interact with different efficiency with the transducers (G_i and G_x) that regulate adenylate cyclase and phospholipase C.

The proposed existence of muscarinic receptor subtypes has attracted increasing attention (1). One hypothesis, based on analogy with the α-adrenergic receptor, is that the muscarinic receptors that inhibit adenylate cyclase and those that stimulate PI hydrolysis may differ. We have previously shown that there are differences in the potency and effectiveness of several muscarinic agonists for eliciting these two biochemical responses in chick heart cells (2). However, neither the classical muscarinic antagonist atropine nor the "M_1-selective" antagonist pirenzepine showed much selectivity for blocking muscarinic effects on cyclic AMP versus PI metabolism in chick heart cells (3). Although it is possible that receptors with different binding properties couple to the two responses, an alternative explanation for the selectivity in the effects of agonists is that there are differences in muscarinic receptor reserve for the two responses. The experiments presented here provide evidence that the carbachol-binding properties of the receptors coupled to the two responses are the same. The data also demonstrate that there is a great deal of receptor reserve for muscarinic receptor coupling to adenylate cyclase and relatively little reserve in the coupling of the muscarinic receptor to phospholipase C. This would explain why there are differences in the potency of carbachol and in the ability of weak agonists such as oxotremorine to regulate cyclic AMP versus PI metabolism.

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Materials and Methods

Cell preparations. Cells were prepared from 13-day-old chick embryo hearts, as described previously (2). Briefly, the cells were enzymatically dispersed in Ca^{2+}, Mg^{2+}-free buffer containing 0.25% trypsin, and heart cells were separated from erythrocytes and cell debris by centrifugation in 30% Percoll. Dissociated cells were assayed in suspension, within 1 hr of preparation.

Receptor inactivation. PrBCM was made up to a concentration of 100 μM in 10 mM sodium phosphate buffer, pH 7.6, and incubated for 60 min at room temperature to allow formation of the active aziridinium ion, as described by Burgen *et al.* (4). The mustard was then added to cells at the desired concentrations and the cells were incubated for 20 min at 30°. The cells were then washed three times by resuspension and centrifugation at 600 × *g* for 5 min. The number of receptors remaining following alkylation was determined by radioli-

ABBREVIATIONS: PI, phosphoinositide; NMS, *N*-methylscopolamine; PSS, physiological salt solution; HEPES, 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid; PrBCM, propylbenzilylcholine mustard.

gand binding assays as described below, using saturating concentrations of [^{3}H]NMS.

Assay conditions. All assays were carried out at 35° in buffered PSS 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. The PSS was buffered to pH 7.4 with 25 mM $NaHCO_3$ (gassed with 95% O_2/5% CO_2) or with 20 mM HEPES (for radioligand binding).

Cyclic AMP assays. Chick heart cells were equilibrated with 100 μM isobutylmethylxanthine for 20 min before addition of ascorbate (vehicle), 3 μM isoproterenol, or 3 μM isoproterenol plus carbachol for 2 min. The assay was terminated and cyclic AMP was purified and assayed by the competitive protein-binding assay as described (2).

PI assays. PI hydrolysis was monitored by measuring [^{3}H]inositol 1-phosphate accumulation in the presence of 10 mM LiCl. PIs were radiolabeled by incubating the cells for 1 hr in PSS containing 5–10 μCi/ml of *myo*-[2-^{3}H]inositol. Cells were assayed at a concentration of approximately 10^7 cells/ml. The assay was terminated 30 min after agonist addition by centrifugation of the cells, sonication in chloroform/methanol, and separation of [^{3}H]inositol 1-phosphate from [^{3}H]inositol by anion exchange chromatography as modified (5) from the method of Berridge *et al.* (6).

Radioligand-binding assays. Binding assays were carried out on intact chick heart cells suspended in HEPES-buffered PSS containing a range of [^{3}H]NMS concentrations (20 pM–1 nM) or with a single concentration 3–6 times the K_D (K_D = 0.18 nM). Incubations were carried out for 60–75 min and were terminated by lysing the cells with hypotonic buffer and filtering on Whatman GF/C filters. Nonspecific binding (that not inhibited by 10 μM atropine) was <25%. Competition curves were analyzed using a weighted nonlinear least squares computer curve-fitting program, LIGAND (7).

Materials. Fertilized White Leghorn chicken eggs were obtained from McIntyre Poultry and Eggs (San Diego, CA). Atropine sulfate, carbamylcholine chloride (carbachol), and oxotremorine were from Sigma Chemical Co. (St. Louis, MO). PrBCM was a gift from Dr. Palmer Taylor, University of California, San Diego. *myo*-[2-^{3}H]Inositol and [^{3}H]NMS were from New England Nuclear (Boston, MA). AG1-X8 anion exchange resin, 100–200 mesh, formate form was from Bio-Rad Laboratories (Richmond, CA).

Results

Intact chick heart cells were incubated with activated PrBCM at concentrations ranging from 30 pM to 1 μM for 20 min. Cells were then washed three times and labeled with a saturating concentration of [^{3}H]NMS for 60 min. Treatment with PrBCM decreased the B_{max} but not the K_D for NMS (not shown). The PrBCM concentration-dependent decrease in specific [^{3}H]NMS binding sites is shown in Fig. 1. In some experiments 5–10% residual [^{3}H]NMS binding remained, even at the highest concentrations of PrBCM. The significance of this residual NMS binding is unclear, but it probably depends on the definition of nonspecific binding that is used. We have therefore subtracted this small fraction of residual binding sites to define the point at which 100% of the receptors are alkylated.

The carbachol concentration-response curve for inhibition of isoproterenol-stimulated cyclic AMP formation is shifted rightward following PrBCM treatment (Fig. 2). In cells treated with 10 nM PrBCM, which reduced binding by 95%, the EC_{50} for carbachol is increased more than 10-fold but carbachol still gives maximal inhibition of cyclic AMP formation. This contrasts with the effect of PrBCM treatment on PI hydrolysis (Fig. 3). Here the predominant effect of receptor inactivation is to reduce the maximal response to carbachol. Thus, following treatment with 10 nM PrBCM, even high concentrations of carbachol fail to cause the maximal inositol phosphate formation seen in untreated cells.

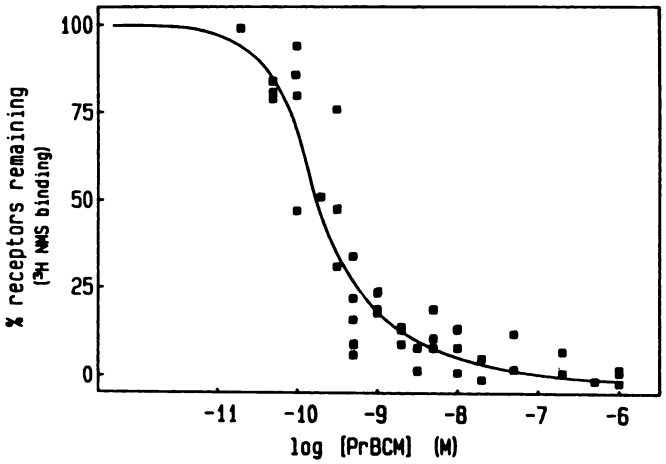

Fig. 1. Decrease in muscarinic receptors in PrBCM-treated cells. Data from eight separate experiments, in which various concentrations of PrBCM were used, are combined here. The percentage of receptors remaining in the PrBCM-treated cells was calculated using 100% as the number of specific [^{3}H]NMS-binding sites in cells incubated without PrBCM (~5000 sites/cell or 170 fmol/mg of protein) and 0% as that in cells treated with a maximally inhibitory concentration of PrBCM.

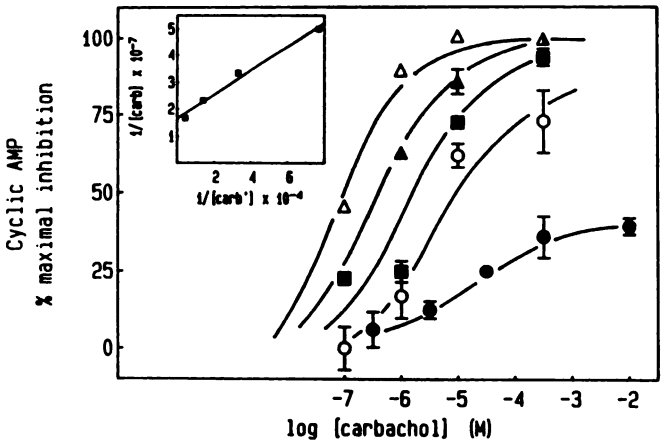

Fig. 2. Effect of PrBCM treatment on concentration response curve for inhibition of cyclic AMP formation. Cells were treated with PrBCM at 0.5 nM (▲), 10 nM (■), 0.5 μM (○), or 1 μM (●), washed, and then challenged with isoproterenol and various concentrations of carbachol. The basal and isoproterenol-stimulated cyclic AMP concentrations were 9.5 and 72.0 pmol of cyclic AMP/mg of protein, respectively. These values were unaffected by PrBCM treatment. In control cells (△), 10 μM carbachol gave maximal inhibition of the response to isoproterenol (to 18.9 pmol/mg of protein). The data are expressed as a percentage of this maximal inhibition seen in control cells. *Inset*: Equiactive doses of carbachol in control cells and cells treated with 1 μM PrBCM were calculated and their reciprocals were plotted. The *straight line* fitted to the data by linear regression analysis had a correlation coefficient of 0.99 and gave values of K_A = 27 μM, $q = 2.2 \times 10^{-3}$.

We previously reported a 100-fold disparity in the potency of carbachol for regulating cyclic AMP versus PI metabolism (2). That disparity in the concentration response curves is markedly diminished following alkylation with PrBCM as shown in Fig. 4. Once receptor reserve is removed by irreversible receptor inactivation, the K_A of the receptor for an agonist can be determined through measurements of biological responses, as described by Furchgott and Bursztyn (8). Equiactive concentrations of agonist before and after receptor inactivation are

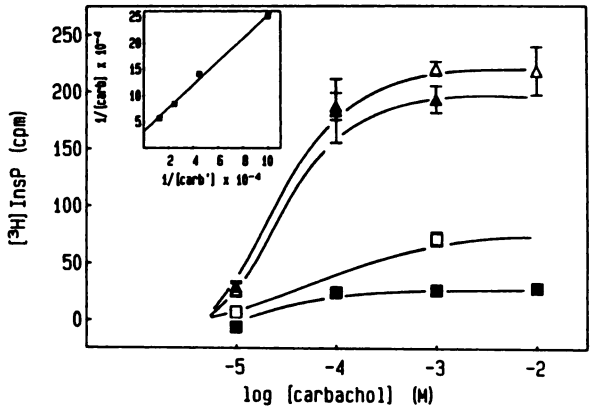

Fig. 3. Effect of PrBCM treatment on concentration-response curve for stimulation of PI hydrolysis. Cells were incubated under control conditions (△) or with PrBCM at 0.5 nM (▲), 5 nM (□), or 10 nM (■), washed, and challenged with carbachol. The basal [^{3}H]inositol phosphate (*[^{3}H]InsP*) formation (~60 cpm) was unaffected by PrBCM treatment and has been subtracted. Thrombin-induced PI hydrolysis was also unaffected by PrBCM treatment, indicating that there is not a nonspecific reduction in the PI response. *Inset*: Equiactive doses of carbachol in control and PrBCM-treated cells were calculated and their reciprocals were plotted. Linear regression analysis gave a plot with a correlation coefficient of 0.99 and gave values of $K_A = 39$ μM, $q = 0.45$.

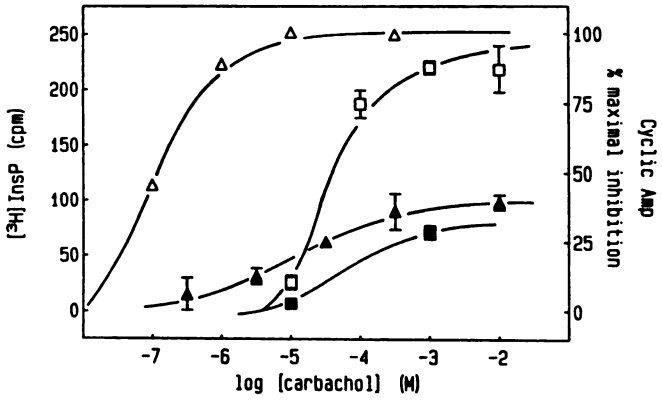

Fig. 4. Dose-response relationships for muscarinic inhibition of cyclic AMP and stimulation of [^{3}H]inositol phosphate (*[^{3}H]InsP*) formation before and after receptor alkylation. In control cells the EC_{50} values for inhibition of cyclic AMP (△) and stimulation of [^{3}H]inositol phosphate formation (□) by carbachol are 0.1 μM and 30 μM, respectively. Following treatment with sufficient PrBCM to decrease the maximal responses (1 μM PrBCM for cyclic AMP, 5 nM PrBCM for [^{3}H]inositol phosphate), the EC_{50} for inhibition of cyclic AMP formation (▲) is approximately 10 μM and that for stimulation of [^{3}H]inositol phosphate formation (■) is approximately 50 μM.

determined and these are plotted as shown in the *insets* to Figs. 2 and 3. The K_A is calculated from the intercept and slope of the regression line. The K_A values thus derived for the receptors regulating the cyclic AMP and PI responses are nearly equivalent, averaging 38 and 39 μM, respectively (Table 1).

Radioligand binding studies were carried out using intact chick heart cells, so that binding parameters could be described under conditions similar to those used to generate the biological responses. The competition of carbachol for [^{3}H]NMS-binding sites in the intact cell is usually best fit by a model in which carbachol binds to all of the [^{3}H]NMS sites with a single affinity.[1] The average K_D for carbachol is 35 μM (Table 1).

[1] When [^{3}H]Quinuclidinylbenzilate is used as a ligand, a second site with much lower affinity for carbachol is also seen (2). This may be explained by [^{3}H]quinuclidinylbenzilate labeling some receptor sites that are not labeled by [^{3}H]NMS; these sites have low apparent affinity for quaternary ligands (8a).

TABLE 1

Dissociation constants for carbachol, calculated from dose response curves after receptor inactivation and from radioligand binding

The functional K_A values were obtained by Furchgott analysis (8) done on two separate experiments. The binding data are the mean ± standard error from three experiments.

Parameter	Dissociation constant (K_A or K_D)
	μM
Inhibition of cyclic AMP formation	27; 50
Stimulation of PI hydrolysis	39; 39
Competition for [^{3}H]NMS-binding sites	35 ± 9

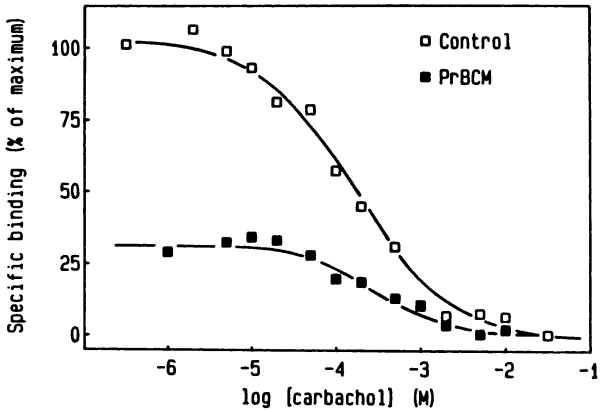

Fig. 5. Carbachol competition for [^{3}H]NMS-binding sites before and after alkylation with 1 nM PrBCM. Cells were incubated in the presence or absence of 1 nM PrBCM, washed, and then assayed for radioligand binding with 0.9 nM [^{3}H]NMS. Both carbachol competition curves were best fit by one-site models which gave K_D values for carbachol of 25 μM in untreated cells (□) and 54 μM in cells treated with PrBCM (■).

Similar values are obtained when assays are carried out at 0°, or under nonequilibrium conditions in which cells are only briefly (30 sec) exposed to agonist (data not shown). A monophasic curve with a similar K_D for carbachol is also obtained following alkylation of approximately 70% of the receptors with PrBCM (Fig. 5). This indicates that the shifts in the concentration-response relationships for the functional responses following PrBCM treatment are not the result of changes in receptor affinity for carbachol.

The K_A and K_D values calculated from the experiments described above are compared in Table 1. The data indicate that, after receptor reserve is removed, one no longer observes differences in apparent affinity of carbachol in the two response systems. Furthermore, the receptor affinities determined in these response systems agree well with the single affinity usually observed in radioligand binding experiments.

Oxotremorine is a full agonist for inhibiting cyclic AMP formation (2, 9) but only a partial agonist for stimulating PI hydrolysis in chick heart cells, 1321N1 astrocytoma cells, or brain (2, 5, 10, 11), or for eliciting a chronotropic response in the chick heart (12). After chick heart cells are treated with 50 nM PrBCM, leading to loss of ~95% of the receptors, oxotremorine becomes a partial agonist and can no longer give maximal inhibition of cyclic AMP formation even at the highest concentration tested (Fig. 6). This contrasts with the lack of a PrBCM-induced change in the cyclic AMP response to carbachol, shown again in Fig. 6 for comparison with oxotremorine. These data demonstrate that the effect of removing receptor reserve is specific for the agonist being tested. They also

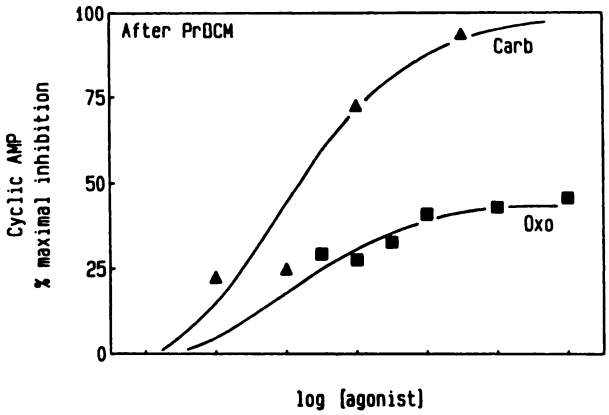

Fig. 6. Effect of PrBCM treatment on inhibition of cyclic AMP formation by carbachol and oxotremorine. In control cells (not shown), carbachol and oxotremorine both inhibit isoproterenol-stimulated cyclic AMP formation to the same extent (see Ref. 2). The maximal inhibitory effect of carbachol or oxotremorine is expressed as 100% maximal inhibition. Under the conditions used here, PrBCM treatment does not decrease the maximal inhibitory response to carbachol, but the maximal inhibition by oxotremorine is reduced to less than 50% of that achieved in control cells. For technical reasons 50 nM PrBCM was used in the oxotremorine experiment, vs. 10 nM in the carbachol experiment. However, the data in Fig. 2 demonstrate that even 500 nM PrBCM would not significantly depress the carbachol curve.

demonstrate that differences in the effect of carbachol and oxotremorine can be seen for the cyclic AMP response (as they were for the PI response) once receptor reserve is removed.

Discussion

The effects of acetylcholine and other cholinergic agonists on muscarinic receptors have classically been studied by examining physiological responses such as smooth muscle contraction (8, 13–15). Muscarinic responses assumed to be more proximal to the muscarinic receptor than contraction, e.g., inhibition of cyclic AMP formation (2, 9, 16), formation of cyclic GMP (17), and stimulation of PI hydrolysis (2, 5, 10, 11, 18, 19) have also been studied. The advent of radioligand binding methodology has provided the most direct means of examining agonist interactions with muscarinic receptors. Radioligand binding studies of muscarinic receptors in membranes, and often those in intact cells or synaptosomes, reveal at least two agonist affinity states (10, 17, 20–22). Accordingly, attempts have been made to equate a particular affinity state (e.g., high or low affinity) with generation of a biochemical response (e.g., cyclic AMP or cyclic GMP formation, PI hydrolysis, contraction) elicited by muscarinic receptor occupation (10, 17, 20–22).

The classical studies of Stephenson (14) established that the potency of an agonist for eliciting smooth muscle contraction response did not equal the affinity of that agonist for the receptor, because potency was also determined by another factor that he termed "efficacy." Even when more proximal biochemical responses are measured, agonist potency (or EC_{50}) will be a function of the affinity of the muscarinic receptors for agonist, but will also contain an efficacy term describing how efficiently receptor occupation initiates the biochemical response.

Stephenson's (14) definition of efficacy was based on the concept that not all agonists are equal in their ability to transduce receptor occupation into response. He distinguished a series of muscarinic agonists of differing efficacies. Such a distinction is directly evident in the comparison of a series of muscarinic agonists for their effects on PI hydrolysis (5, 10, 11). In every system examined, oxotremorine and other weak agonists fail to stimulate PI hydrolysis to the same extent as carbachol. For other responses [i.e., chronotropic effects on the chick heart (12), contraction of the ileum (15), and inhibition of cyclic AMP formation (2)], oxotremorine appears as effective as carbachol. In each of these systems, however, the maximal response to oxotremorine can be decreased under conditions of receptor inactivation that do not decrease the maximal effect of carbachol (Refs. 12 and 15; Fig. 6).

Efficacy, or the relationship between occupancy and response, differs not only with the agonist but also with the tissue (8). The differences between tissues can be understood in terms of the total number of available functional receptors, referred to as differences in "receptor reserve." An extension of the notion that there are differences between tissues is that differences in receptor reserve may be determined by the effector to which the receptor couples. This appears to be the case for the systems examined here. In PrBCM-treated chick heart cells, carbachol can still fully inhibit cyclic AMP formation following inactivation of >95% of total muscarinic receptors. In contrast, a modest decrease in receptor number markedly attenuates carbachol-stimulated inositol phosphate production. Thus, there appears to be greater than 95% reserve in the coupling of carbachol-occupied muscarinic receptors to adenylate cyclase, whereas there is a more linear relationship between muscarinic receptor occupancy and production of a PI response. We suggest that it is this difference in receptor reserve that explains the disparity in the potency of carbachol and the effectiveness of oxotremorine for activating the two biochemical responses.

The molecular basis for differences in "receptor reserve" cannot yet be defined. Although the receptor states mediating the two responses in chick heart cells do not appear different based on their affinity for carbachol (Table 1) or for antagonists (3), it remains possible that the receptor populations that interact with the two effector systems are not the same. The number of functional receptors available to interact with phospholipase C (or with the putative guanine nucleotide-binding protein, G_x, that regulates PI hydrolysis) may therefore be less than the number of receptors available to inhibit adenylate cyclase.[2] Alternatively, the same population or subtype of receptors might mediate either response [just as β-adrenergic receptors appear able to interact with G_s or G_i (24)], but the probability of a functional interaction between the muscarinic receptor and G_x may be low relative to the probability of a muscarinic receptor-G_i interaction. Clearly it will be necessary to define the molecular entities involved, their stoichiometry, and their mode of interaction, before the phenomena we describe can be understood in molecular terms.

Our conclusion that there is relatively little reserve in receptor coupling to PI hydrolysis is consistent with the early suggestions of Michell and others of a close relationship between receptor occupancy and the extent of PI hydrolysis (18, 19, 25, 26). Functionally, receptor reserve can also occur distal to the

[2] We suggest that a G protein regulates PI hydrolysis because guanosine 5′-*O*-(3-thiotriphosphate) stimulates [^{3}H]inositol phosphate formation in permeabilized chick heart cells (23). It is unlikely that G_i is the G protein involved in the PI response because pertussis toxin does not inhibit PI hydrolysis in chick heart cells (23a).

biochemical responses examined here. Thus, despite the occurrence of little reserve in receptor coupling to PI hydrolysis, one might achieve maximal mobilization of calcium with less than maximal inositol trisphosphate formation, or the maximal increase in cytosolic calcium may be greater than needed to cause a full contractile response. In the 1321N1 astrocytoma cell, although there appears to be little muscarinic receptor reserve for stimulation of PI hydrolysis, submaximal PI hydrolysis is sufficient to maximally activate a more distal response, specifically, the calcium-dependent activation of phosphodiesterase (27). In the final analysis, then, the lack of reserve in receptor coupling to PI hydrolysis may be overridden by the capacity of the cell to translate small second messenger signals into full physiological responses.

The K_A values for carbachol, calculated from the two functional assays by the method of Furchgott (8), are very close to the K_I values that we calculate from radioligand binding experiments carried out on these cells. Nathanson (28) calculated a similar value for the single class of carbachol-binding sites in his studies of [^{3}H]NMS binding to intact chick heart cells. These intact cell dissociation constants are most like those of the "low-affinity" carbachol binding site seen in chick heart membranes (2, 21, 28), which range from 10 to 50 μM. It is also informative that similar K_A values (between 10 and 40 μM) were obtained in studies examining carbachol-induced contractions following receptor inactivation in rabbit fundus (8), guinea pig ileum (15), or rabbit ciliary body (29), or examining carbachol-stimulated secretion in pancreatic acini (22). The similarity in the carbachol dissociation constants reported throughout the literature, including those obtained in our direct comparison of receptors coupled to cyclic AMP and phospholipid metabolism, is striking. These data suggest that muscarinic receptors that couple to these varied responses are not markedly different in the affinity with which they bind carbachol. Our data suggest, however, that muscarinic receptors differ in the efficiency with which agonist binding leads to receptor interactions with particular guanine nucleotide-binding proteins.

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