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Receptor Reserve in the Calcium-Dependent Cyclic AMP Response of Astrocytoma Cells to Muscarinic Receptor Stimulation: Demonstration by Agonist-Induced Desensitization, Receptor Inactivation, and Phorbol Ester Treatment

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SUMMARY

Activation of muscarinic cholinergic receptors on 1321N1 human astrocytoma cells results in attenuation of cyclic AMP accumulation, apparently as a consequence of increases in phosphoinositide hydrolysis, Ca^{2+} mobilization, and the activation of a Ca^{2+} calmodulin-regulated phosphodiesterase. Preincubation of these cells with carbachol for 30–90 min resulted in a 15–30-fold shift to the right of the concentration effect curves for carbachol or methacholine for attenuation of cyclic AMP accumulation, with no change occurring in the maximal effect observed with either agonist. In contrast, preincubation with carbachol for 30–90 min resulted in an essentially complete loss of effects on cyclic AMP accumulation of the muscarinic receptor agonists oxotremorine, arecoline, and bethanechol. In contrast to carbachol and methacholine, oxotremorine, arecoline, and pilocarpine were much less effective inducers of desensitization. Inactivation of muscarinic receptors with propylbenzylcholine mustard, or preincubation of cells with 4β-phorbol 12β-myristate 13α-acetate, which has been shown to markedly decrease the phosphoinositide response of 1321N1 cells to cholinergic agonists, produced differential effects on the cyclic AMP response to carbachol and oxotremorine that were similar to those observed after preincubation with carbachol. The data can be explained by the presence of "reserve" in the series of steps that result in muscarinic receptor-mediated activation of phosphodiesterase. That is, it is proposed that carbachol and methacholine mobilize much more Ca^{2+} than is necessary for maximal activation of phosphodiesterase, whereas oxotremorine and several other muscarinic receptor agonists do not.

Target cell responsiveness decreases during prolonged exposure of cells to hormones or neurotransmitters. This receptor desensitization or down-regulation has been described in the greatest detail for peptide hormone receptors (1, 2) and the β-adrenergic receptor/adenylate cyclase system (3, 4). However, the phenomenon occurs at other receptors, including the muscarinic cholinergic receptor. Richelson and co-workers (5–7) and Galper and co-workers (8–10) have reported the most extensive descriptions of muscarinic receptor desensitization. These studies have focused on the cyclic GMP response to cholinergic stimuli in cultured neuroblastoma cells (5–7) and the response of cultured heart cells to prolonged exposure to muscarinic receptor agonists (8–10).

Stimulation of muscarinic cholinergic receptors on 1321N1 human astrocytoma cells attenuates cyclic AMP accumulation (11, 12). This effect is not a result of inhibition of adenylate cyclase but, rather, results from calcium-dependent activation of phosphodiesterase leading to increased cyclic AMP degradation (12–14). Several lines of evidence suggest that these responses occur through a sequence of events initiated by a muscarinic receptor-stimulated increase in phosphoinositide turnover (15), leading to calcium mobilization (15) and then to activation of a calcium, calmodulin-regulated phosphodiesterase (16). Although the muscarinic receptor agonists carbachol and oxotremorine are equally efficacious as activators of phosphodiesterase (12, 17), carbachol is a much more efficacious stimulator of phosphoinositide breakdown and Ca^{2+} mobilization in 1321N1 cells (15, 18). In addition, the capacity of cholinergic agonists to stimulate cyclic AMP degradation decreases markedly with extended exposure of these cells to carbachol but not to oxotremorine (14).

In the present study loss of the cyclic AMP response to muscarinic receptor agonists was examined following agonist pretreatment, receptor alkylation, and treatment with a phor-

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ABBREVIATIONS: ^{3}H-QNB, ^{3}H-quinuclidinylbenzilate; PMA, 4β-phorbol 12β-myristate 13α-acetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBCM, propylbenzylcholine mustard; $K_{0.5}$, concentration of agonist that produced 50% of the maximal effect.

bol ester. These perturbations diminish signal transduction by different means, yet each resulted in a similar pattern of loss of muscarinic receptor-mediated activation of phosphodiesterase. Loss of responsiveness to agonists that produce large increases in phosphoinositide hydrolysis and Ca^{2+} mobilization occurred as a decrease in apparent affinity for reduction in cyclic AMP levels that was followed by a loss in maximal effect. In contrast, loss of responsiveness to agonists that cause only small increases in phosphoinositide hydrolysis and Ca^{2+} mobilization occurred as a loss of maximal effect with no notable effects on apparent affinity. The results are consistent with the presence of significant "reserve" in the pathway by which muscarinic receptor occupancy is translated into increased cyclic AMP degradation. Furthermore, the data may be generally representative of changes in the response of a multistep hormone-response system in which agonists differ in their efficacy for activation of an initial event.

Methods

Cell culture. The 1321N1 human astrocytoma cells were grown as previously described (12). Cells were plated at a density of 10,000–20,000 cells/cm^2 and were grown for 5–7 days in 12-well dishes.

Analysis of cyclic AMP accumulation. Cyclic AMP levels were measured using the ^{3}H-adenine prelabeling method as previously described (12). Briefly, the growth medium was aspirated and replaced with 1 ml of 25 mM Hepes-buffered Eagle's medium (pH 7.4). Depending on the experiment, the Eagle's medium contained carbachol (or another muscarinic receptor agonist) or ^{3}H-adenine (1 μCi) or both. Labeling of cells with ^{3}H-adenine was always for 60–75 min. For drug challenges, the medium was aspirated, the cells were rapidly washed two times with Hepes-buffered Eagle's medium (2 ml; 20°), and isoproterenol (10 μM) or isoproterenol (10 μM) plus a muscarinic receptor agonist was added. Drug challenges were usually for 5 min at 37° and were terminated by aspiration of the drug-containing medium and addition of 1.0 ml of 5% trichloroacetic acid containing 0.5 mM cyclic AMP. ^{3}H-ATP and ^{3}H-cyclic AMP were separated by chromatography on Dowex 50-X8 and alumina columns (12); recovery of cyclic AMP was monitored by UV spectrometry at 259 nm.

Quantitation of muscarinic cholinergic receptors. Muscarinic receptors were quantitated in cell lysates with ^{3}H-QNB as we have previously described (17).

Data presentation. All assays were carried out in triplicate and quadruplicate and all experiments were carried out at least three times. The data are presented as mean ± standard error of the indicated number of experiments or as mean values of replicates from an individual experiment that is representative of a series of experiments.

Results

Preincubation of 1321N1 cells with carbachol for 75 min produced two prominent changes in cellular responsiveness (Fig. 1). As we have described previously (14), isoproterenol-stimulated cyclic AMP accumulation was increased in carbachol-pretreated cells. This effect is not agonist specific, e.g., the stimulatory effect of prostaglandin E_1 also increases (Ref. 14 and data not shown), and apparently involves an increase in hormone responsiveness of adenylate cyclase (14). Carbachol reduced cyclic AMP accumulation by 50–75% in control 1321N1 cells (Fig. 1). Following preincubation of cells with 100 μM carbachol for 75 min, the attenuation of cyclic AMP accumulation produced by a maximally effective concentration of carbachol was similar to control levels. However, the $K_{0.5}$ for carbachol was increased approximately 30-fold. Little change

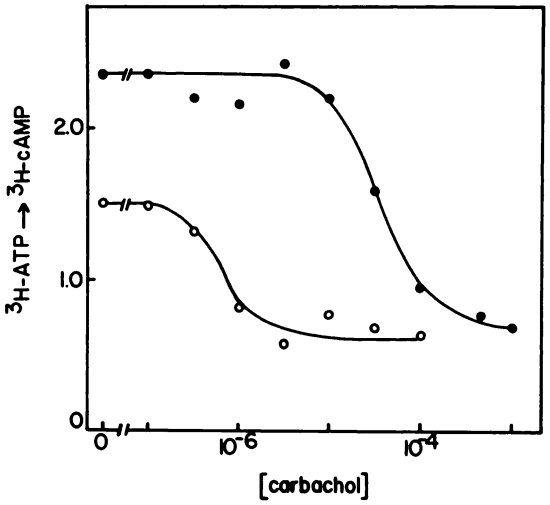

Fig. 1. Agonist-induced change in responsiveness of 1321N1 cells to carbachol. 1321N1 cells were incubated with (●) or without (○) 100 μM carbachol for 75 min, then washed free of agonist, and rechallenged for 5 min with 10 μM isoproterenol and the indicated concentrations of carbachol. The data are presented as percentage conversion of ^{3}H-ATP to ^{3}H-cyclic AMP and are representative of results obtained in seven similar experiments.

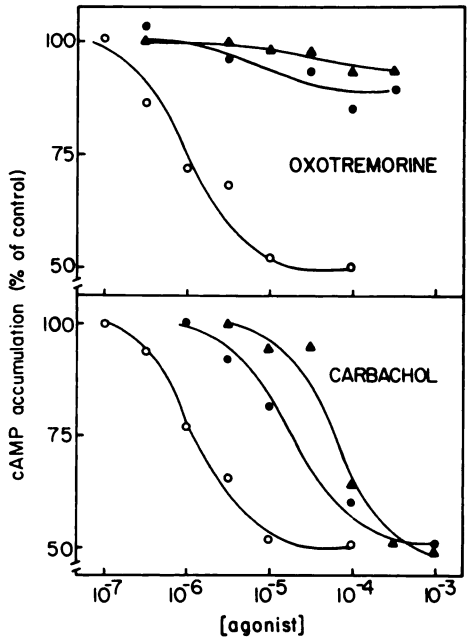

Fig. 2. Comparison of agonist-induced changes in responsiveness to carbachol and oxotremorine. 1321N1 cells were incubated in the absence (○) of carbachol or in the presence of 100 μM carbachol for 30 (●) or 90 (▲) min. The cells then were washed free of drug and rechallenged for 5 min with 10 μM isoproterenol and the indicated concentrations of agonist (*top*: oxotremorine, *bottom*: carbachol). The data are presented as the percentage of cyclic AMP accumulation observed in the presence of isoproterenol alone under each condition: 3.4, 5.3, and 5.4% conversion of ^{3}H-ATP to ^{3}H-cyclic AMP for the control, 30-min carbachol pretreatment, and 90-min carbachol pretreatment conditions, respectively. The results are representative of those obtained in four similar experiments.

in the number of muscarinic receptors measured with ^{3}H-QNB occurs during a 75-min incubation with carbachol (14).

The effect of preincubation with carbachol on subsequent responsiveness to carbachol and oxotremorine is illustrated in Fig. 2. The carbachol-induced increase in response to isoproterenol (see Fig. 2 legend for actual values) is not apparent in

this figure since the data are presented as percentage of isoproterenol-stimulated cyclic AMP accumulation for each condition. Again, preincubation of cells with carbachol for 30 or 90 min resulted in a shift to the right of the concentration effect curve for carbachol with little change in maximal response (Fig. 2, *bottom*). Preincubation with carbachol caused similar changes in the concentration effect curve for methacholine (data not shown). In contrast, under the same conditions there was a large decrease in the maximal effect of oxotremorine on cyclic AMP accumulation (Fig. 2, *top*), i.e., the stimulatory effects of oxotremorine on cyclic AMP degradation were lost (Ref. 14 and data not shown). No reproducible effect of carbachol pretreatment on the $K_{0.5}$ of oxotremorine for reduction of cyclic AMP accumulation was observed.[3]

Although incubation of 1321N1 cells with 100 μM carbachol for 90 min or less only decreased the apparent affinity, i.e., increased the $K_{0.5}$, of carbachol for attenuation of cyclic AMP accumulation, prolonged exposure eventually resulted in a loss of the maximal effect of carbachol (Fig. 3). Thus, after a 24-hr preincubation with carbachol, no muscarinic receptor-stimulated activation of phosphodiesterase occurred (Fig. 3 and data not shown). The time course of the agonist-induced loss of maximal effect of carbachol closely paralleled the loss of muscarinic receptors from the cell. That is, we have reported previously (14) that, following a lag of approximately 1 hr, muscarinic receptors, detected in lysates using ^{3}H-QNB, are lost from 1321N1 cells with a $t_{1/2}$ of approximately 6 hr. Long-term treatment of 1321N1 cells with 100 μM carbachol also results in a parallel loss in the number of ^{3}H-*N*-methylscopolamine-binding sites on intact cells and in the maximal phosphoinositide response to carbachol (19).

The capacity of carbachol for inducing loss of responsiveness to a series of agonists was tested. As illustrated in Fig. 4,

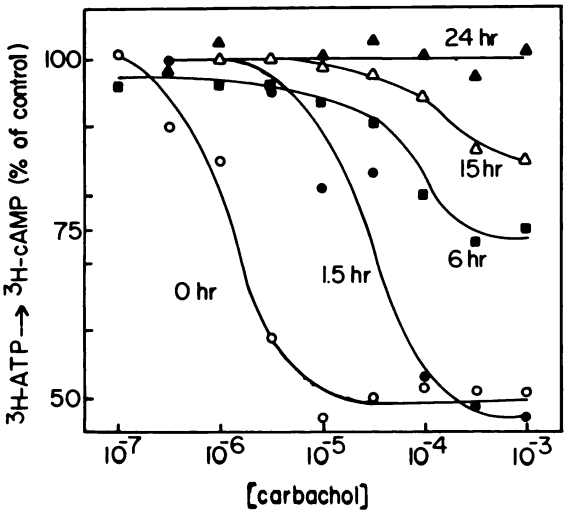

Fig. 3. Responsiveness to carbachol after prolonged exposure to agonist. Cells were incubated with 100 μM carbachol for 0 (○), 1.5 (●), 6 (■), 15 (△), or 24 (▲) hr, washed free of drug, and rechallenged for 5 min with 10 μM isoproterenol and the indicated concentrations of carbachol. The results are representative of those obtained in four similar experiments.

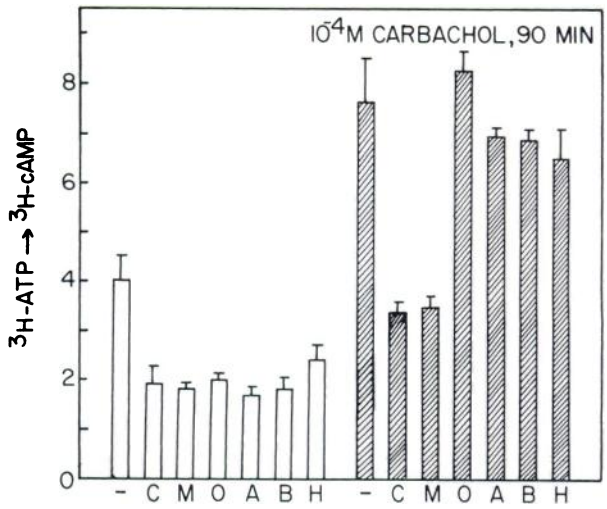

Fig. 4. Carbachol-induced change in responsiveness to various cholinergic agonists. 1321N1 cells were pretreated with (▨) or without (□) 100 μM carbachol for 90 min, washed free of drug, and rechallenged with 10 μM isoproterenol alone or 10 μM isoproterenol plus 100 μM carbachol (*C*), methacholine (*M*), oxotremorine (*O*), arecoline (*A*), bethanechol (*B*), or histamine (*H*). The results are presented as percentage conversion of ^{3}H-ATP to ^{3}H-cyclic AMP and are the mean ± standard error of three experiments.

carbachol, methacholine, oxotremorine, arecoline, and bethanechol all reduced cyclic AMP accumulation to approximately the same level in control cells. After pretreatment of cells with 100 μM carbachol for 90 min, there was a nearly complete loss of responsiveness to 100 μM oxotremorine, arecoline, and bethanechol. This effect represented a loss of maximal effect of agonist since higher concentrations of each of these agonists were also ineffective in reducing cyclic AMP accumulation (data not shown). In contrast to the results obtained with these three cholinergic agonists, 100 μM carbachol and methacholine still inhibited cyclic AMP accumulation by 60% after carbachol pretreatment.

Since activation of H_1-histamine receptors on 1321N1 cells results in an increase in inositol phosphate formation, Ca^{2+} mobilization, and degradation of cyclic AMP (20), it was of interest to examine the effects of carbachol preincubation on the capacity of histamine to attenuate cyclic AMP accumulation. As illustrated in Fig. 4, the effects of histamine were essentially lost after preincubation with carbachol. Carbachol-induced loss of histamine responsiveness occurred as a reduction of maximal effect with little change in apparent affinity (data not shown).

We previously reported that oxotremorine is much less effective than carbachol for inducing desensitization of muscarinic receptor-mediated effects on cyclic AMP accumulation in 1321N1 cells (14). The effects of preincubation (90 min) with oxotremorine and other agonists on the capacity of carbachol to reduce cyclic AMP accumulation are illustrated in Fig. 5. As illustrated above, preincubation of 1321N1 cells with maximally effective concentrations of carbachol or methacholine resulted in an increase in isoproterenol-stimulated cyclic AMP accumulation and a loss of inhibitory activity of a low (10 μM) concentration of carbachol. In contrast, preincubation with maximally effective concentrations[4] of arecoline and oxotre-

[3] Numerous experiments were carried out to determine whether pretreatment of cells with carbachol for shorter times resulted in an increase in $K_{0.5}$ of oxotremorine *prior* to a loss of maximal effect. The only reproducible modification in the concentration effect curve of oxotremorine for reduction of cyclic AMP levels was a decrease in maximal effect.

[4] Concentrations of agonists that maximally stimulate (18) inositol phosphate formation (100 μM for all agonists except bethanechol, which was at a concentration of 1000 μM) were used. Essentially the same results were obtained when this experiment was carried out using all agonists at a concentration of 500 μM.

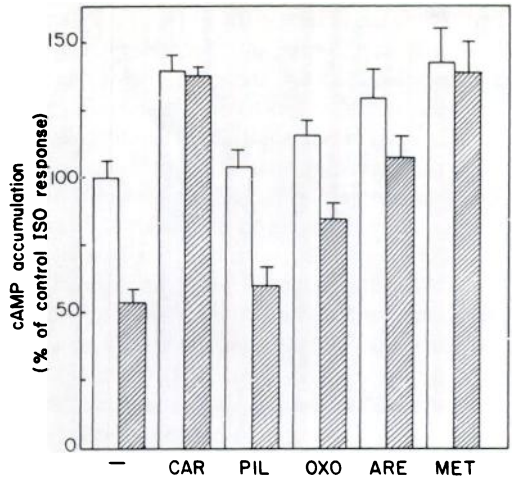

Fig. 5. Effects of pretreatment with various muscarinic cholinergic receptor agonists on carbachol-induced inhibition of cyclic AMP accumulation. 1321N1 cells were incubated for 90 min in the absence of drug or in the presence of 100 μM carbachol (*CAR*), pilocarpine (*PIL*), oxotremorine (*OXO*), arecoline (*ARE*), or methacholine (*MET*). The cells were then washed free of drug and rechallenged for 5 min with 10 μM isoproterenol (□) or 10 μM isoproterenol plus 10 μM carbachol (▨). The results are the mean ± standard error of three experiments.

morine resulted in a partial loss of the inhibitory effect of 10 μM carbachol, whereas preincubation of 1321N1 cells with pilocarpine, which is less than 5% as efficacious as carbachol for stimulation of phosphoinositide breakdown (18), caused no effect on the response of 1321N1 cells to either isoproterenol or carbachol.

One explanation for the observation that high concentrations of carbachol and methacholine still decrease cyclic AMP accumulation after preincubation with carbachol is that carbachol and methacholine cause phosphoinositide breakdown and Ca^{2+} mobilization in excess of that necessary to maximally activate phosphodiesterase. This phenomenon can be described as an example of "receptor reserve" in the action of carbachol and methacholine on cyclic AMP accumulation. Oxotremorine and other weak agonists lose their effectiveness after carbachol pretreatment because they normally elicit minimal phosphoinositide breakdown and Ca^{2+} mobilization. If the hypothesis of reserve in the action of carbachol is correct, other means of reduction of muscarinic receptor responsiveness also should differentially affect responses to carbachol and oxotremorine. Two approaches for production of such a modification in muscarinic receptor responsiveness were tested. First, PBCM was utilized to inactivate muscarinic cholinergic receptors. Preincubation of 1321N1 cells with 10 nM PBCM for 30 min resulted in an 80% loss of muscarinic receptors measured using ^{3}H-QNB in cell lysates (Fig. 6). This effect was long-lasting (no recovery of receptors occurred in 6 hr) and occurred with no change in the K_d of ^{3}H-QNB for the remaining receptors. When inhibition of cyclic AMP accumulation in response to carbachol or oxotremorine was measured in PBCM-pretreated cells, results similar to those observed after carbachol pretreatment were obtained. That is, the carbachol concentration-effect curve shifted approximately 20-fold to the right, whereas there was a complete loss of responsiveness to oxotremorine (Fig. 7).

Orellana *et al.* (21) have reported recently that treatment of 1321N1 cells with a phorbol ester results in loss of capacity of muscarinic receptors for stimulation of inositol phosphate formation or Ca^{2+} mobilization without a decrease in receptor

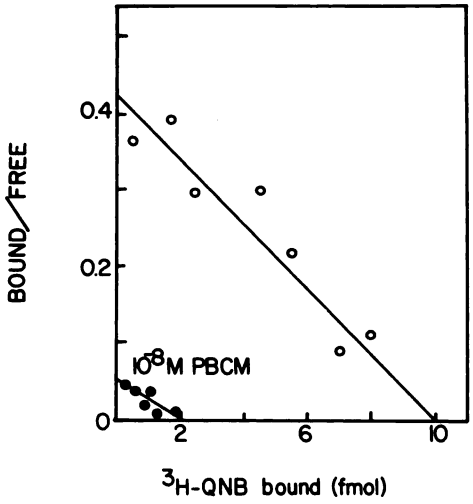

Fig. 6. PBCM-induced decrease in the density of muscarinic receptors. 1321N1 cells were incubated in the absence (○) or presence (●) of 10 nM PBCM for 30 min. The cells were then washed five times with Hepes-Eagle's buffer and lysed hypotonically, and ^{3}H-QNB binding assays were carried out using lysates as described in Methods. The data are plotted as Scatchard plots of saturation binding isotherms with the amount of ^{3}H-QNB specifically bound plotted on the *abscissa* and the ratio of specifically bound to free radioligand (liters × 10^3) plotted on the *ordinate*. The K_d values were 25 and 50 pM for control and PBCM-treated cells, respectively. The results are representative of those obtained in five similar experiments.

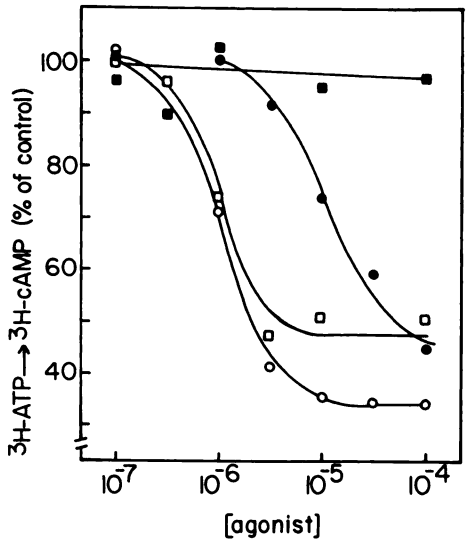

Fig. 7. Effect of pretreatment with PBCM on responsiveness to carbachol and oxotremorine. Cells were incubated in the absence (○, □) or presence (●, ■) of 10 nM PBCM for 30 min, washed three times with Hepes-Eagle's buffer over a period of 30 min, and rechallenged with 10 μM isoproterenol and the indicated concentrations of carbachol (○, ●) or oxotremorine (□, ■). The results are representative of those obtained in four similar experiments.

number. Experiments measuring inositol phosphate formation in a cell-free preparation suggest that the modification of responsiveness occurs through an effect at the level of the putative guanine nucleotide-regulatory protein that couples hormone receptors to activation of phospholipase C.[5] As illustrated in Fig. 8, incubation of 1321N1 cells with PMA also caused a loss of effect of carbachol on cyclic AMP accumulation.

[5] S. Orellana and J. H. Brown, submitted for publication.

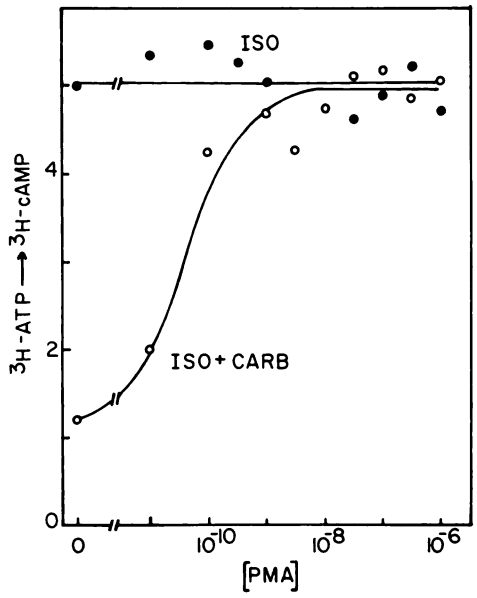

Fig. 8. Concentration-dependent effect of PMA on muscarinic receptor-mediated inhibition of cyclic AMP accumulation. 1321N1 cells were incubated for 10 min with the indicated concentrations of PMA and then challenged with 10 μM isoproterenol (*ISO*, ●) or 10 μM ISO + 100 μM carbachol (*ISO+CARB*, ○) for 5 min. PMA was also present during the drug challenge. The results are presented as the percentage conversion of ^{3}H-ATP to ^{3}H-cyclic AMP and are representative of eight similar experiments.

This effect was half-maximal at a concentration of PMA of 0.3–1.0 nM and was maximal at 10 nM. The changes in agonist responsiveness after treatment with PMA were similar to the changes observed after carbachol pretreatment of 1321N1 cells. The concentration-effect curve for carbachol was shifted 30-fold to the right prior to any loss of response to a maximally effective concentration of agonist (Fig. 9, *right*); the change in oxotremorine responsiveness mainly occurred as a loss of efficacy (Fig. 9, *left*). Whereas 1 nM PMA did not decrease the maximal effect of carbachol, the effect of oxotremorine on cyclic AMP accumulation was lost after treatment with this concentration of phorbol ester.

Discussion

Cyclic AMP accumulation in 1321N1 cells is regulated in part by a Ca^{2+}, calmodulin phosphodiesterase (16). Activation of muscarinic cholinergic receptors on these cells activates this phosphodiesterase apparently as a secondary response to the formation of inositol 1,4,5-trisphosphate, which in turn mobilizes Ca^{2+} from internal stores. Thus, measurement of attenuation of cyclic AMP accumulation in these cells represents analysis of a phenomenon that is several steps beyond the initial agonist-muscarinic receptor interaction. As has been described elegantly by Strickland and Loeb (22) and Swillens and Dumont (23), multistep hormone response systems such as this will very predictably display discrepancies between receptor occupancy curves and biological response curves. In addition, responses to "second messengers," e.g., activation of physiological events by cyclic AMP (24) or, in this case, activation of phosphodiesterase by Ca^{2+}, may express "receptor reserve" in the sense that much more second messenger is generated than is necessary to activate the enzyme maximally.

It is not yet possible to describe accurately agonist-muscarinic receptor occupancy curves since determination of the affinity of agonists in whole cells under conditions identical to those used for measurement of biochemical responses is not possible. Nonetheless, previously published data (17) indicate that the $K_{0.5}$ values of carbachol and methacholine for inhibition of cyclic AMP accumulation in 1321N1 cells are approximately 30-fold less than their K_i values determined in ^{3}H-QNB competition binding experiments with washed membranes from the same cells in the presence of GTP. Although it could be argued that these analyses were made under vastly different conditions, it is notable that the $K_{0.5}$ and K_i values are not discrepant for the muscarinic receptor agonists oxotremorine, arecoline, and bethanechol. Thus, whereas a relatively linear relationship between receptor occupancy and biological response holds for oxotremorine, arecoline, and bethanechol, carbachol and methacholine appear to stimulate phosphodiesterase maximally at low receptor occupancy, indicating the presence of receptor reserve in the final biological response to these agonists. Viewed another way, the agonist-, PBCM-, or PMA-induced increase in $K_{0.5}$ for reduction of cyclic AMP accumulation reached the apparent affinity of carbachol (30–50 μM) or methacholine (5–10 μM) for stimulation of inositol phosphate formation prior to any loss of maximal effect. In contrast, even in the absence of drug pretreatment, the $K_{0.5}$ values of oxotremorine (approximately 3 μM) and the other partial agonists for reduction of cyclic AMP accumulation were similar to the $K_{0.5}$ values of these agonists for stimulation of

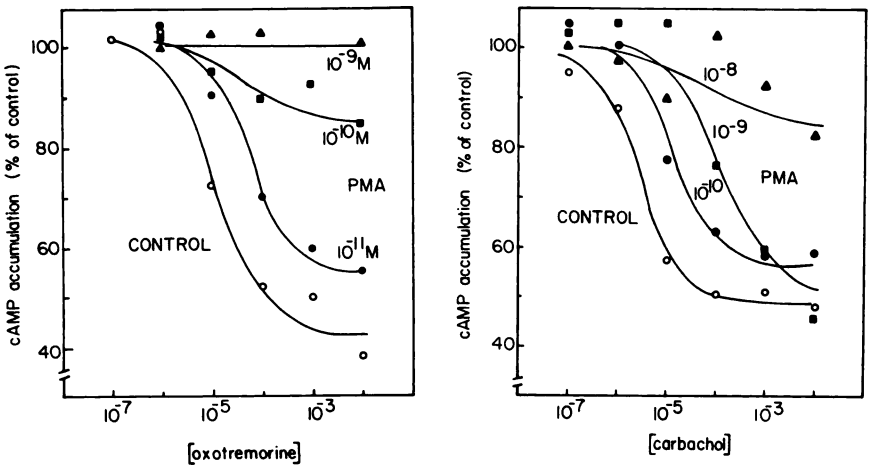

Fig. 9. PMA-induced changes in cellular responsiveness to carbachol and oxotremorine. Cells were incubated with the indicated concentrations of PMA for 10 min and then challenged with 10 μM isoproterenol + various concentrations of carbachol (*right*) or oxotremorine (*left*) for 5 min. PMA was present during drug challenge. Results are representative of four similar experiments.

inositol phosphate formation. Following drug treatment, maximal effects of these agonists on cyclic AMP levels decreased with no change in $K_{0.5}$. Finally, oxotremorine, arecoline, and bethanechol are much less efficacious as stimulators of phosphoinositide breakdown and Ca^{2+} mobilization than are carbachol or methacholine (18). In spite of this difference in efficacies for the initial biochemical response to muscarinic receptor stimulation, the maximal activation of phosphodiesterase and attenuation of cyclic AMP accumulation are the same for the five agonists. These data suggest that carbachol and methacholine mobilize much more Ca^{2+} than is necessary for maximal activation of phosphodiesterase, whereas oxotremorine, bethanechol, and arecoline mobilize Ca^{2+} to an extent that is barely sufficient for maximal activation.

The relative changes in responsiveness to different cholinergic agonists subsequent to carbachol pretreatment support the ideas discussed above. That is, because carbachol and methacholine are capable of increasing intracellular Ca^{2+} levels in excess of that necessary to maximally stimulate the Ca^{2+}, calmodulin phosphodiesterase, decreases in their capacity to stimulate Ca^{2+} mobilization would first be observed as a decrease in their apparent potency rather than in their maximal effect on cyclic AMP accumulation. A reduction in the maximal effect of carbachol and methacholine would only occur after the Ca^{2+} response decreased to a level insufficient to maximally activate the phosphodiesterase. Conversely, the change in response to compounds such as oxotremorine that produce relatively small increases in intracellular Ca^{2+}, e.g., concentrations of Ca^{2+} that are similar to those that maximally activate the phosphodiesterase, mainly would be expressed as a loss of maximal effect of the agonist for attenuation of cyclic AMP accumulation. These ideas also are supported by the experiments with PBCM and the phorbol ester PMA. That is, reduction in receptor number by PBCM or reduction in the capacity of agonists to stimulate inositol phosphate formation and Ca^{2+} mobilization by treatment with PMA both resulted in the predicted change in effects of carbachol and oxotremorine on cyclic AMP accumulation.

Whereas the activation of phosphodiesterase by carbachol or methacholine occurs with considerable "receptor reserve," this is apparently not the case for the effects of these agonists on inositol phosphate formation. That is, agonist-induced loss of muscarinic receptors from 1321N1 cells occurs coincidentally with a loss of maximal effects of carbachol on inositol phosphate formation (19). Similar results have been obtained in chick heart cells where inactivation of muscarinic receptors with PBCM results in loss of maximal effects of carbachol on inositol phosphate formation.[6] In contrast, the inhibition of adenylate cyclase by carbachol in the same tissue expresses receptor reserve in that inactivation of muscarinic receptors results in large increases in $K_{0.5}$ values for agonist prior to any loss of maximal effect.

Definition of the mechanism by which cholinergic responses desensitize in 1321N1 cells was not a goal of this study, although the work complements previous findings in this regard. Masters *et al.* (19) have reported that breakdown of phosphoinositides and formation of inositol trisphosphate are sustained for up to 75 min of continuous exposure of 1321N1 cells to carbachol. Thus, the large loss of the phosphodiesterase response to cholinergic stimuli that occurs during a 75-min exposure to carbachol apparently does not occur due to major changes of responsiveness at the level of the receptor. Loss of effects of inositol trisphosphate on Ca^{2+} release or depletion of a hormone-sensitive pool of Ca^{2+} might account for loss of the effects of cholinergic agonists on the cyclic AMP response of these cells. Either of these mechanisms also would explain desensitization of the response to histamine. Histamine acts through an H_1-histamine receptor to stimulate phosphoinositide breakdown, Ca^{2+} mobilization, and phosphodiesterase activation in 1321N1 cells (20). The effects of histamine and carbachol on any of these responses are not additive, suggesting that a common mechanism of action is shared. Thus, carbachol-induced loss of responsiveness to inositol trisphosphate or depletion of a hormone-responsive calcium pool would result in heterologous desensitization which would extend to the effects of histamine on cyclic AMP accumulation. The loss of response to histamine occurred as a decrease in maximal response with no change in apparent affinity (data not shown). This would be predicted by the arguments made thus far, since histamine, like oxotremorine, is only 20–30% as efficacious as carbachol for stimulation of phosphoinositide breakdown (20). Either of the above mechanisms also would explain why muscarinic receptor agonists are not all equally effective inducers of desensitization. Thus, carbachol and methacholine, which cause the largest increase in inositol trisphosphate formation, and calcium mobilization would inhibit subsequent responses to a greater extent than oxotremorine and other partial agonists that marginally elevate inositol trisphosphate levels.

Results from this study emphasize a relationship that has not always been appreciated in the analysis of hormone and drug action and in consideration of the ramifications of changes in cellular responses to agonists. First, in multicomponent response systems changes in a maximal effect at an early step in a second messenger cascade may be reflected as changes in apparent potency for producing a subsequent biological response. To our knowledge, this point was first made in an elegant way by Drummond *et al.* (25). These workers demonstrated that maximal cyclic AMP-mediated activation of phosphorylase by catecholamines in C6 glioma cells occurred at a concentration of agonist that produced only 6% of the maximally attainable elevation of cyclic AMP concentration. A 3-hr preincubation of these cells with isoproterenol resulted in a 60% loss of maximal effect of isoproterenol on cyclic AMP accumulation, with no change occurring in apparent affinity. Nonetheless, isoproterenol still fully activated phosphorylase in these "desensitized" C6 glioma cells, but the concentration-effect curve for agonist was shifted 15-fold to the right. This "receptor reserve" in hormone or drug action has obvious therapeutic importance in that agents that are seemingly equally efficacious in producing a final pharmacological response may be very different in maximal effectiveness for production of some initial or intermediate response. Thus, modification of the degree of response at one of these earlier steps could result in a complete loss of pharmacological response to one agent while only decreasing the apparent affinity of another agent that is more efficacious in producing one of the earlier responses.

Completely quantitative analysis of the cholinergic response of 1321N1 cells is not yet possible. Although we have provided evidence for a relationship of phosphoinositide breakdown to

[6] J. H. Brown and D. Goldstein, manuscript in preparation.

Ca^{2+} mobilization in 1321N1 cells, the physiological activities of the isomers (1,3,4 versus 1,4,5) of inositol trisphosphate (26), of inositol 1,3,4,5-tetrakisphosphate (27), and of inositol 1:2-cyclic 4,5-trisphosphate (28) are not fully defined. In addition, although $^{45}Ca^{2+}$ efflux studies suggest the existence of a depletable hormone-sensitive pool of Ca^{2+}, changes in intracellular Ca^{2+} levels in response to hormone receptor agonists or Ca^{2+} ionophores have not been quantitated directly in 1321N1 cells. To this end, high pressure liquid chromatographic analysis of individual inositol phosphate accumulation, as well as analysis of intracellular Ca^{2+} levels using fluorescent probes, is being developed for 1321N1 cells. Comparison of data obtained using this methodology, together with measurement of phosphodiesterase activity in intact 1321N1 cells, should allow us to make quantitative statements about the response relationships operative in this multistep system.

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