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Alpha Adrenergic and Cholinergic-Muscarinic Regulation of Adenosine Cyclic 3',5'-Monophosphate Levels in the Rat Parotid

YORAM ORON, JOAN KELLOGG, AND JOSEPH LARNER

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903

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SUMMARY

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(−)-Isoproterenol caused a dose-dependent rise in cyclic AMP concentration in rat parotid slices. Preincubation of rat parotid slices with carbamylcholine or an *alpha* adrenergic agonist, methoxamine, markedly inhibited the rise of cyclic AMP following stimulation with a *beta* adrenergic agonist, (−)-isoproterenol. This effect of carbamylcholine was blocked by preincubation with atropine and the effect of methoxamine was blocked by preincubation with phenoxybenzamine. The effects of methoxamine or carbamylcholine were partly reversed by subsequent addition of phentolamine or atropine. Carbamylcholine produced an almost maximal inhibition when added simultaneously with (−)-isoproterenol, while methoxamine required more than 8 min of preincubation to achieve its maximal effect. Carbamylcholine was active either in the absence of exogenous Ca^{++} or in the presence of Ca^{++} and the divalent cation ionophore A23187. Methoxamine was inactive in the absence of added Ca^{++} and failed to reduce further the (−)-isoproterenol stimulated concentrations of cyclic AMP in the presence of Ca^{++} and A23187. Methoxamine, A23187, and carbamylcholine each caused a shift to the right in (−)-isoproterenol dose-response curve. The non-hyperbolicity of the dose response curve to (−)-isoproterenol prevented a simple analysis of the resulting inhibition patterns. Both carbamylcholine and methoxamine produced their effects in the presence of cyclic AMP diesterase inhibitors. Carbamylcholine had little, if any, effect on the rate of disappearance of cyclic AMP. These findings strongly indicate a different mechanism of action for the *alpha* adrenergic and cholinergic agonists in the rat parotid, which previously have been reported to act via independent receptors, to produce apparently identical physiological and biochemical effects.

INTRODUCTION

In numerous tissues cell metabolism is altered by a hormone-stimulated activation of membranous adenylate cyclase and the rise of cyclic AMP[1] concentration subsequently triggers various control mechanisms through the activation of cyclic AMP-dependent protein kinase. In many cells the *beta* adrenergic receptor co-exists with the *alpha* adrenergic receptor. The natural adrenergic neurotransmitter, nor-

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[1] The abbreviations used are: cyclic AMP, adenosine cyclic 3',5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; KRB, Krebs-Ringer bicarbonate buffer; SDS, sodium dodecyl sulfate.

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epinephrine, activates both adrenergic receptors simultaneously. Many cells also contain additional hormone receptors. The complex interactions of two or more activated receptors determine the physiological modulation of cell function.

The rat parotid gland is a convenient model system for the elucidation of several of these interactions. There are three independent and pharmacologically distinct receptors in the parotid acinar cell. A *beta* adrenergic receptor controls the secretion of exportable proteins via the rise in cyclic AMP concentration. Activation of the *alpha* adrenergic receptor leads to massive and rapid K^+ release mediated, most probably, by the rise in the concentration of intracellular Ca^{++}. A cholinergic-muscarinic receptor controls the same physiological response via an apparently identical mechanism (1, 2).

It has been reported that stimulation of the cholinergic receptor in the rat parotid markedly inhibits the rise in cyclic AMP concentration elicited by *beta* adrenergic agonists (3, 4). It has been demonstrated by Batzri *et al.* that a simultaneous activation of both *alpha* and *beta* adrenergic receptors in the rat parotid leads to lower levels of cyclic AMP than those following *beta* adrenergic stimulation alone (5). This *alpha* adrenergic inhibition of cyclic AMP accumulation due to *beta* adrenergic stimulation was further documented by Butcher *et al.* (6). There are numerous reports of cyclic AMP levels reduced in certain tissues by either *alpha* adrenergic (7–12) or cholinergic (13–16) receptors.

The purpose of the present study was to determine the interactions of *alpha* adrenergic and cholinergic receptors with the *beta* adrenergic system reflected by the cyclic AMP concentration changes. The role of Ca^{++} was particularly studied because of its crucial importance in *alpha* adrenergic and cholinergic mechanism of action in the rat parotid.

MATERIALS AND METHODS

Wistar male rats (150–200 g) were used in all experiments. The rats were fasted overnight prior to sacrifice. Parotid glands were dissected from connective tissue and blood vessels, minced with scissors and washed with 5 ml of Krebs-Ringer bicarbonate buffer solution (128 mM NaCl, 2.5 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl and 1.5 mM KH_2PO_4, pH 7.4) containing 10 mM β-hydroxybutyrate. Gland slices were incubated in 5 ml of the same buffer for 30–60 min, washed in 5 ml buffer and resuspended in 5 ml fresh buffer. Slices equivalent to 1–3 mg protein were transferred at 30 sec intervals to polypropylene test tubes containing 1 ml of KRB buffer containing appropriate additions. Following a 15 min incubation period, effectors were added, usually in a volume of 10 μl. At all stages, the slices were shaken vigorously at 37° and gassed with oxygen:carbon dioxide (95%:5%). Incubation was terminated by adding trichloroacetic acid to a final concentration of 5% with immediate homogenization with a polytron tissue mincer. The homogenates were centrifuged for 10 min × 12,000 *g*. The supernatant volume was adjusted to 2 ml and assayed for cyclic AMP. The protein pellets were dissolved in 2.5% solutions SDS in 0.5 N NaOH, adjusted to pH 5–9, and protein was assayed according to Lowry *et al.* (17).

Cyclic AMP was determined by manual radioimmunoassay or automated radioimmunoassay (18, 19). Potassium was determined by atomic absorption spectrophotometry. When potassium release was measured, slices equivalent to one gland (15–18 mg protein) were suspended in 1 ml of KRB buffer solution containing half the concentration of potassium in the standard buffer. Epinephrine was purchased from Winthrop; isobutyl-methyl-xanthine, (−)-isoproterenol, atropine and carbamylcholine from Sigma Chemical Co.; phenoxybenzamine from Smith, Kline & French; SQ 20009 from Squibb & Sons; methoxamine from Burroughs-Wellcome; phentolamine from Ciba-Geigy. The divalent ionophore A23187 was a gift from Eli Lilly Company. All other chemicals were of analytical grade.

All experiments were performed at least three times in quadruplicate. Results of representative experiments are presented as mean values ± S.E.M. In some experiments absolute values of cyclic AMP meas-

urements were converted to relative values (% of control or % of maximal response) for the sake of clarity or for pooling the results of several experiments (the cyclic AMP values resulting from isoproterenol stimulation varied considerably between individual experiments). The S.E.M. bars represent the real standard error of the mean when multiplied by fraction of the control or maximal response and by the mean value of the control or maximal response point (100%). Difference between two incubation conditions was assumed to be significant when $p < 0.05$ according to Student's paired t-test throughout.

RESULTS

Cyclic AMP rise in response to isoproterenol. The synthetic *beta* adrenergic agonist (−)-isoproterenol caused a dose-dependent rise in cyclic AMP concentration in rat parotid slices (Fig. 1). The Eadie-Hofstee plot was nonlinear (see Fig. 1, inset). The Hill plot yielded a Hill-coefficient value of 0.8 (not shown).

Alpha adrenergic and cholinergic-muscarinic inhibition of the rise of cyclic AMP concentration elicited by (−)-isoproterenol. Carbamylcholine (30 μM), when added before (−)-isoproterenol (2 μM), reduced the rise in cyclic AMP concentration by an average of 70%. This effect of carbamylcholine was completely abolished by the muscarinic blocker, atropine (10 μM) (Fig. 2A).

The specific alpha adrenergic agonist, methoxamine (20 μM) likewise reduced the rise in cyclic AMP concentration by an average of 60% when added before (−)-isoproterenol (2 μM). This effect of methoxamine was completely blocked by either phentolamine (20 μM) or phenoxybenzamine (10 μM) (Fig. 2B). The addition of both carbamylcholine and methoxamine did not result in higher inhibition than that obtained with either agent alone (not shown). Atropine, phentolamine, and phenoxybenzamine by themselves had little, if any, effect on the rise in cyclic AMP stimulated by (−)-isoproterenol (Fig. 2C). In the rat parotid slice system, methoxamine was a weak alpha adrenergic agonist, causing the release of K^+ in concentrations above 50 μM (not shown). Both the alpha adrenergic and the cholinergic effects were partially reversed when phentolamine (20 μM) or

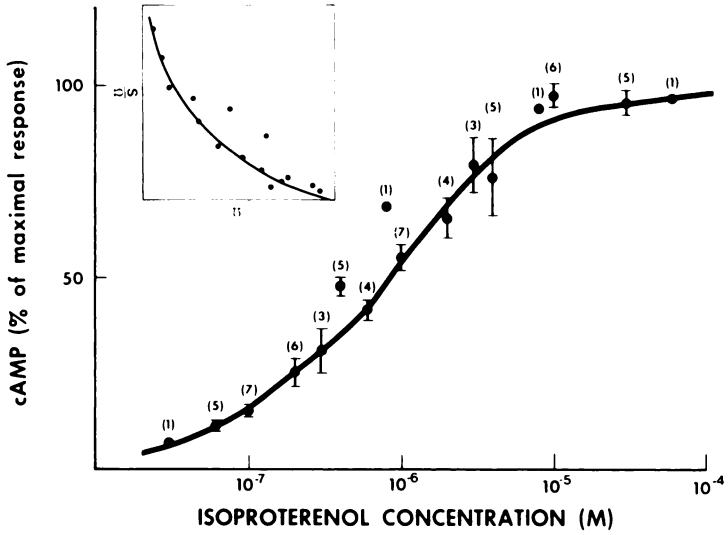

FIG. 1. *(−)-isoproterenol stimulation of cyclic AMP production*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. Incubation was terminated 2 min after the addition of (−)-isoproterenol. The results were converted to relative stimulation (% of maximal response). Values of 11 experiments were pooled. The numbers in brackets denote the number of individual determinations. The mean maximal response was 182.6 ± 23 pmoles cyclic AMP/mg protein at (−)-isoproterenol concentration of 0.1 mM.

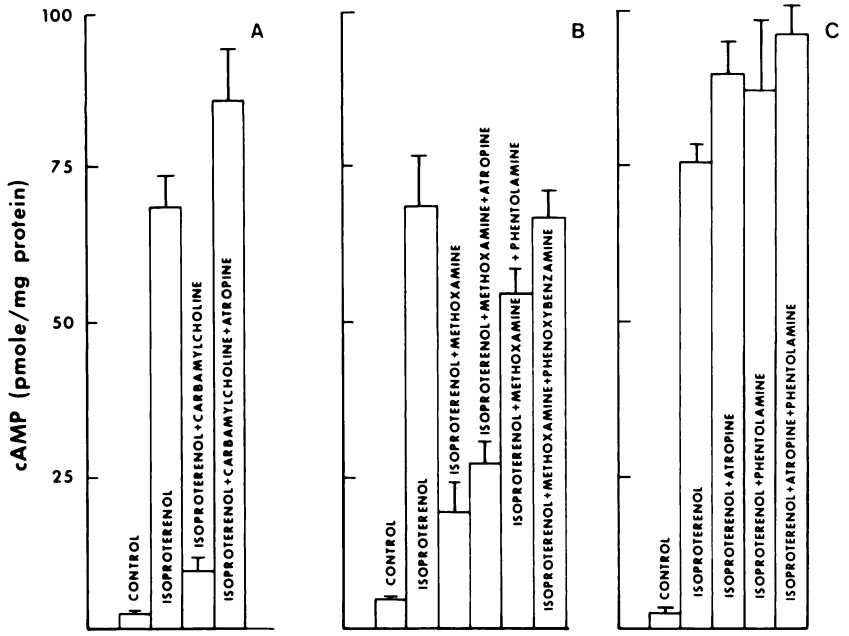

FIG. 2. *The effect of alpha adrenergic and cholinergic agonists and antagonists on (−)-isoproterenol stimulation of cyclic AMP production*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. After 10 min of incubation, agonists and antagonists were added and incubated for 20 min, then isoproterenol was added and the incubation was terminated 2 min later. Drugs were used in the following concentrations: (−)-isoproterenol, 2 μM; carbamylcholine, 30 μM; atropine, 10 μM; methoxamine, 20 μM; phentolamine, 20 μM; phenoxybenzamine, 10 μM.

atropine (10 μM) were added after the addition of methoxamine (20 μM) or carbamylcholine (20 μM) and the slices were preincubated for additional 20 min prior to the stimulation by (−)-isoproterenol (Fig. 3).

The *alpha* adrenergic effect on cyclic AMP concentrations could be demonstrated with epinephrine, which activated both *alpha* and *beta* adrenergic receptors. Epinephrine at various concentrations caused a dose-dependent rise in cyclic AMP levels. Addition of 10 μM phenoxybenzamine enhanced the epinephrine stimulation of cyclic AMP rise at 5 min, presumably by blocking the *alpha* adrenergic action of the hormone (the effect of phenoxybenzamine was less pronounced at 2 min). The *alpha* adrenergic effect of epinephrine on cyclic AMP concentrations was less at high epinephrine concentration (20 μM) (Fig. 4).

The dose response to carbamylcholine and methoxamine. The effect of carbamylcholine on cyclic AMP rise produced by (−)-isoproterenol was dose dependent and was maximal at the concentration of 40 μM (Fig. 5). Methoxamine also produced dose-dependent reduction in cyclic AMP concentrations (Fig. 5), which did not saturate even at concentrations greater than 100 μM. Since this effect of methoxamine, when added in a concentration above 50 μM, was not fully reversed by low concentrations of the *alpha* adrenergic blockers, phentolamine and phenoxybenzamine, it most probably represents inhibition of the *beta* adrenergic response by nonspecific binding of methoxamine to the *beta* adrenergic receptors.

Time course of the action of carbamylcholine and methoxamine. Carbamylcholine produced its maximal effect almost immediately when added together with (−)-isoproterenol. The extent of the inhibition changed little with time of incubation with carbamylcholine, up to 58 min prior to the addition of (−)-isoproterenol (Fig. 6A). Methoxamine, on the other hand, had very little immediate effect when added together with (−)-isoproterenol. The inhibition

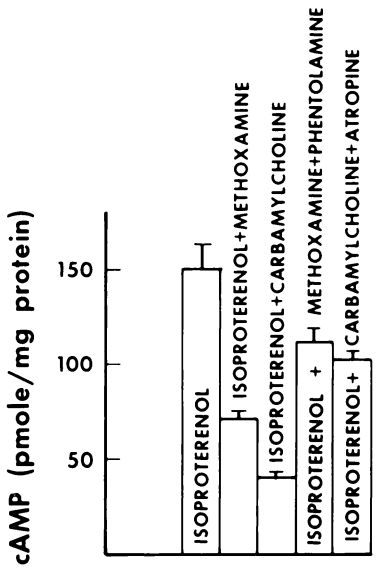

FIG. 3. *The reversibility of the action of methoxamine and carbamylcholine*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. Phentolamine (20 μM) and atropine (10 μM) were added 18 min after the addition of methoxamine (20 μM) or carbamylcholine (20 μM) and the slices incubated for additional 20 min before challenge with (−)-isoproterenol (2 μM) and termination 2 min later.

reached a maximum when methoxamine was added 18 min prior to the addition of (−)-isoproterenol (Fig. 6B).

The pattern of alpha adrenergic and cholinergic inhibition of cyclic AMP response to (−)-isoproterenol. Preliminary experiments indicated that sufficiently high concentrations of (−)-isoproterenol (50 μM) could overcome the inhibition of cyclic AMP formation stimulated by *alpha* adrenergic receptor activation (not shown). To pursue this point further, we studied in detail the dose response to (−)-isoproterenol in the presence of submaximal concentrations of either carbamylcholine (0.3 μM) or methoxamine (5 μM). Both agonists caused a shift in the dose-response curve to the right (Fig. 7 A, B). Both displayed a small degree of inhibition in the lower range of (−)-isoproterenol concentrations (up to 0.7 μM for carbamylcholine and up to 4 μM for methoxamine). In those regions both exhibited an apparent competitivity with (−)-isoproterenol (i.e., diminished inhibition at higher (−)-isoproterenol concentrations). At the higher range of (−)-isoproterenol concentrations both exhibit larger and almost constant inhibition. The pattern of inhibition cannot be analyzed in simple kinetic terms because of the complex shape of the (−)-isoproterenol dose-response curve.

Ca^{++} and the effects of carbamylcholine and methoxamine. Exogenous Ca^{++} is absolutely required for *alpha* adrenergically and cholinergically stimulated K^+ efflux. The action of *alpha* adrenergic and cholinergic agonists can be arbitrarily divided into two components: a component independent of exogenous Ca^{++}, which we labeled primary, and a secondary component, which is entirely dependent on exogenous Ca^{++}, is the result of Ca^{++} influx into the cell, and can be mimicked by artificially increasing the intracellular Ca^{++} and bypassing cell membrane receptors. Receptor-mediated increase in the incorporation of $^{32}P_i$ into parotid phosphatidylinositol is an example of the first class of events (20). Ca^{++}-mediated K^+ efflux is an example of a secondary event. It was of interest to determine

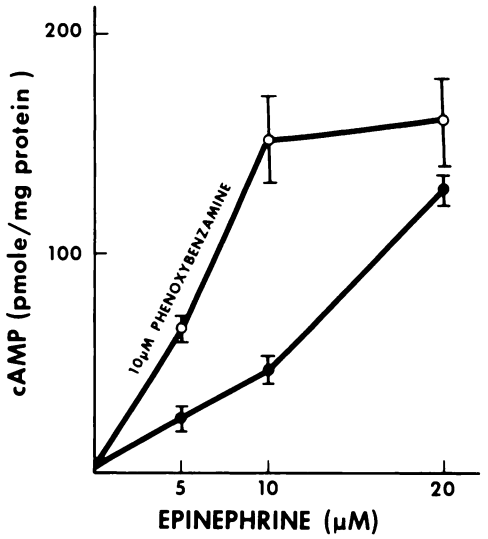

FIG. 4. *The effect of phenoxybenzamine on epinephrine stimulation of cyclic AMP production*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. Phenoxybenzamine (10 μM) was added after 10 min of incubation, followed by epinephrine 15 min later. Incubation was terminated 5 min after the addition of epinephrine.

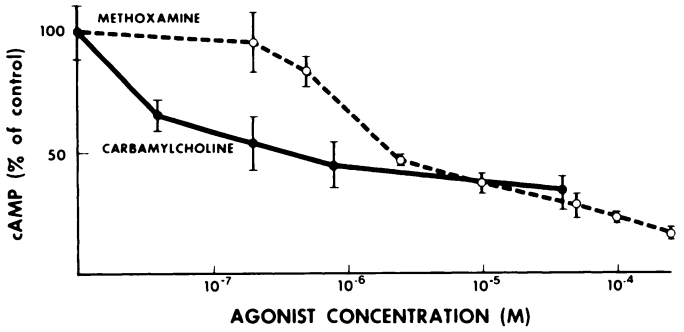

FIG. 5. *Dose-response curves to carbamylcholine and methoxamine*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. Carbamylcholine or methoxamine was added after 10 min incubation, followed by (−)-isoproterenol (2 μM) 20 min later. Incubation was terminated 2 min after the addition of (−)-isoproterenol. Results are expressed as % of control ((−)-isoproterenol alone; 112 pmoles/mg in the carbamylcholine experiment, 75 pmoles/mg in the methoxamine experiment).

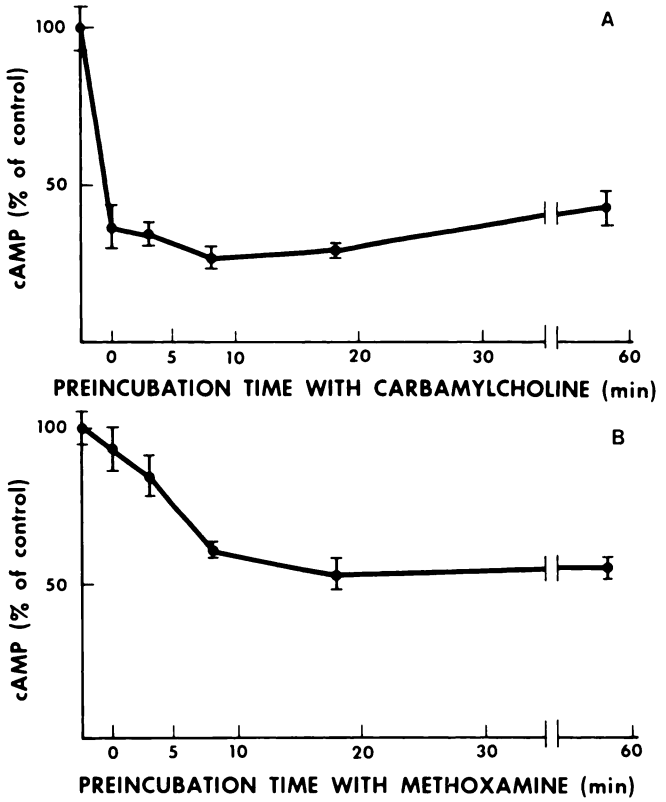

FIG. 6. *The effect of incubation time with carbamylcholine or methoxamine on cyclic AMP production stimulated with (−)-isoproterenol*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. All systems were incubated for 58 min, then (−)-isoproterenol (2 μM) was added and incubation terminated 2 min later. Carbamylcholine (30 μM) or methoxamine (30 μM) were added at various times prior to the addition of (−)-isoproterenol or together with (−)-isoproterenol (0 min preincubation time). Results are expressed as % of control ((−)-isoproterenol alone) represented by the point on the abscissa.

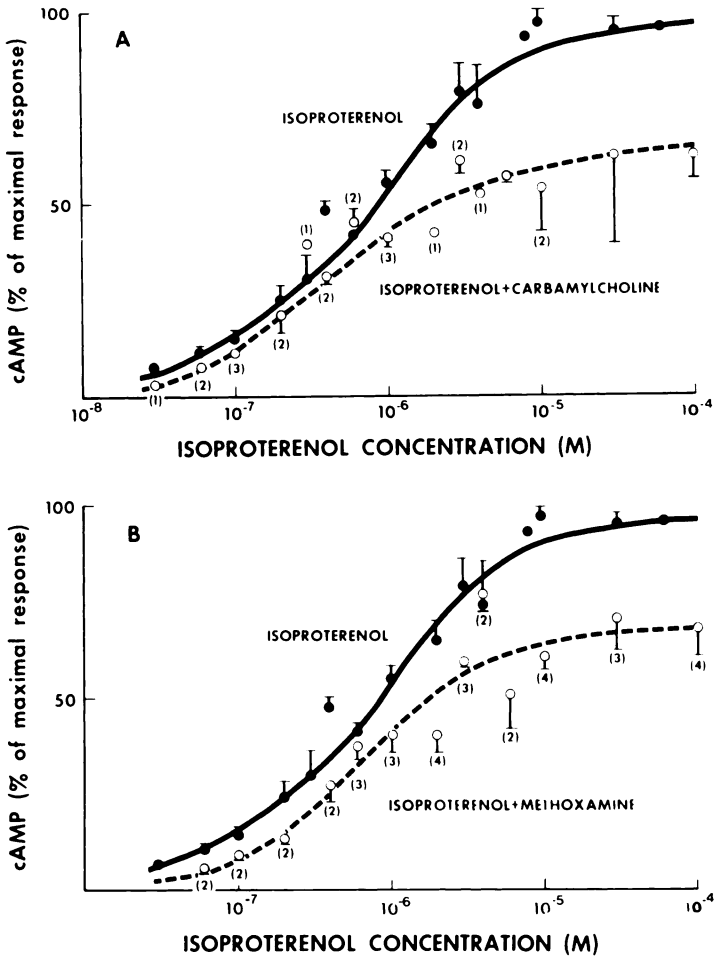

FIG. 7. *The effects of methoxamine and carbamylcholine on the dose-response to (−)-isoproterenol*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. After 10 min incubation, methoxamine (5 μM) or carbamylcholine (0.3 μM) were added. (−)-isoproterenol was added 18 min later and the incubation terminated 2 min after the addition of (−)-isoproterenol. The dose-response curve to (−)-isoproterenol was taken from Fig. 1. Values of 4 experiments with methoxamine and 3 experiments with carbamylcholine were pooled. The numbers in brackets denote the number of individual determinations performed at each (−)-isoproterenol concentration.

whether the *alpha* adrenergic and the cholinergic inhibition of cyclic AMP accumulation belong to one of the above categories.

Carbamylcholine lowered cyclic AMP concentration in the absence of Ca^{++} and in the presence of EGTA (Fig. 8A). Methoxamine, on the other hand, had little, if any, effect on cyclic AMP concentration elicited by (−)-isoproterenol in the absence of Ca^{++} and in the presence of EGTA (Fig. 8C).

The independence of exogenous Ca^{++} of the action of carbamylcholine could be only apparent, since it can be argued that the action of carbamylcholine on cyclic AMP concentrations either requires very low concentrations of Ca^{++} or utilizes intracellular Ca^{++} stores. In order to answer this question, parotid slices were flooded with Ca^{++} by incubating them for 20 min in the presence of 2.5 mM Ca^{++} and a specific divalent cation ionophore A23187 (10 μg/ml). The incubation with A23187 markedly lowered cyclic AMP accumulation fol-

lowing the addition of (−)-isoproterenol. Carbamylcholine further lowered cyclic AMP concentrations by approximately 50% (Fig. 8B). Under the same conditions, methoxamine failed to lower significantly cyclic AMP concentrations (Fig. 8D). The dose-response curve to (−)-isoproterenol in the presence of A23187 (10 μg/ml) was very similar to that obtained in the presence of methoxamine (not shown).

The influence of carbamylcholine on the time course of cyclic AMP disappearance. In order to determine whether carbamylcholine acts on cyclic AMP phosphodiesterase, the following experiment was performed. Slices of rat parotid glands were incubated with (−)-isoproterenol (2 μM). After 3 min a sample was withdrawn and simultaneously 10 μM propranolol, or 10 μM propranolol and 40 μM carbamylcholine, were added. Samples were withdrawn at various times and analyzed for cyclic AMP. Cyclic AMP disappearance followed first order kinetics with $t_{1/2}$ of approximately 1 min. There was little, if any, effect of carbamylcholine on the rate of cyclic AMP disappearance when added simultaneously with propranolol (not shown).

The effect of carbamylcholine and methoxamine in the presence of cyclic AMP phosphodiesterase inhibitors. The addition of cyclic AMP phosphodiesterase inhibitors isobutyl-methyl-xanthine (0.5 mM) or SQ 20009 (10 μM) markedly increased the cyclic

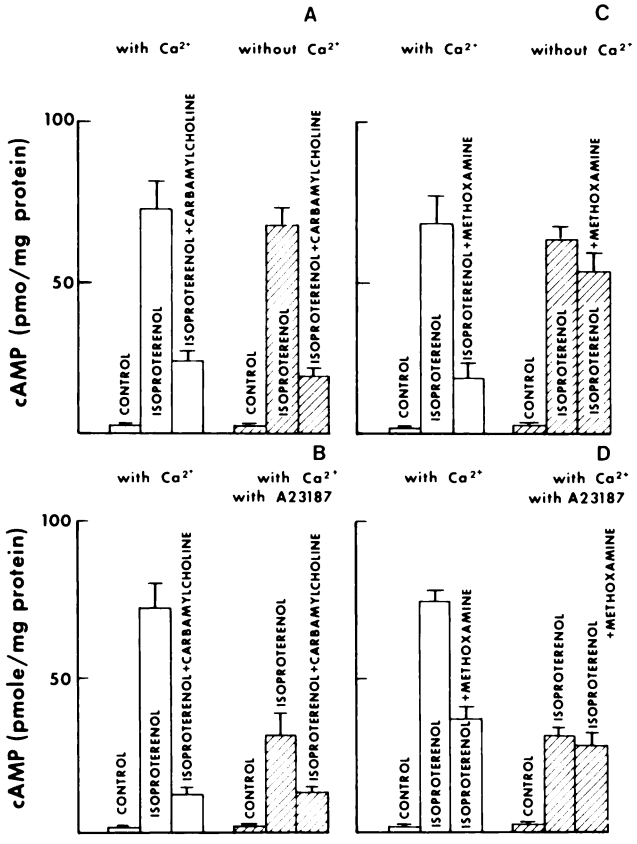

FIG. 8. *The requirement for Ca^{++} for the effects of carbamylcholine and methoxamine*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. Slices were blotted and transferred to complete medium, Ca^{++}-free medium containing 0.2 mM EGTA, or complete medium containing A23187 (10 μg/ml). After 20 min agonists were added, followed 20 min later by (−)-isoproterenol (2 μM). The incubation was terminated 2 min later. A23187 was diluted into KRB buffer from 0.1 M solution in dimethyl sulfoxide. The control systems were exposed to the same concentration of dimethyl sulfoxide.

AMP concentrations after stimulation with (−)-isoproterenol (2 μM). Both carbamylcholine (30 μM) and methoxamine (30 μM) produced a significant decrease in cyclic AMP concentrations in the presence of either isobutyl-methyl-xanthine or SQ 20009 (Fig. 9A, B).

DISCUSSION

1. (−)-Isoproterenol stimulation of cyclic AMP accumulation. (−)-Isoproterenol caused a dose-dependent rise in cyclic AMP accumulation in rat parotid slices. The half-maximal stimulation occurs at (−)-isoproterenol concentration of 0.85 μM which corresponds rather well with the values derived from data of Harper and Brooker (4) (approximately 0.7 μM). The data of Butcher *et al.* (22) yield values of 0.2–0.5 μM, although they do not include the higher concentrations of (−)-isoproterenol in their curves. On closer examination, however, the (−)-isoproterenol dose-response curve is not hyperbolic, the Eadie-Hofstee plot is nonlinear and the apparent Hill coefficient is 0.8. It is of interest to note that several laboratories have reported adenylate cyclase, which exhibited negative cooperativity, particularly in respect to antagonist binding (e.g., Limbird and Lefkowitz (23)).

2. The pharmacology of cyclic AMP modulation. Our data confirm the existence of two distinct and pharmacologically defined receptors in the rat parotid that exert control over the rise of cyclic AMP elicited by the stimulation of the *beta* adrenergic receptor. Carbamylcholine markedly inhibited the accumulation of *cyclic* AMP and its action was abolished by the muscarinic blocker atropine. We could not demonstrate an almost complete inhibition of cyclic AMP accumulation, as reported by Butcher *et al.* (3), even at higher concentrations of carbamylcholine. The average value obtained for saturating concentrations of carbamylcholine was 70% inhibition. Similar findings were reported by Harper and Brooker (4) using norepinephrine as a *beta* adrenergic agonist. The specific *alpha* adrenergic agonist methoxamine caused similar inhibition in cyclic AMP accumulation, which was blocked by the *alpha* adrenergic inhibitors phentolamine and phenoxybenzamine. The regulation of cyclic AMP concentrations by methoxamine has been previously reported by Butcher *et al.* (6).

Although the effects of carbamylcholine and methoxamine were completely abolished by incubation of the tissue slices with specific blockers, the action of the agonists was only partially reversed by adding the blockers after prior incubation with the agonists.

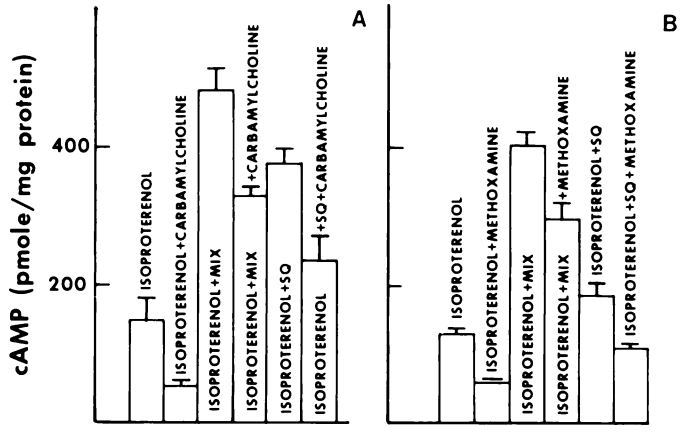

FIG. 9. *The effects of carbamylcholine and methoxamine in the presence of cyclic AMP phosphodiesterase inhibitors*

Rat parotid slices were incubated as described in MATERIALS AND METHODS. After 15 min incubation drugs were added to the following concentrations: carbamylcholine and methoxamine, 30 μM; isobutyl-methyl-xanthine (MIX), 0.5 mM; SQ-20009 (SQ), 10 μM. After 12 min (−)-isoproterenol (2 μM) was added and the incubation terminated 2 min later.

The *alpha* adrenergic action of a mixed-adrenergic agent, epinephrine, could be demonstrated by incubating parotid slices with and without phenoxybenzamine. Phenoxybenzamine promoted cyclic AMP accumulation due to epinephrine. Higher concentrations of epinephrine abolished the effect. Similar data were reported by Butcher *et al.* (22) for norepinephrine. At the concentrations of (−)-isoproterenol and phenoxybenzamine used in our studies we did not, however, observe significant changes in (−)-isoproterenol stimulation of cyclic AMP accumulation by phenoxybenzamine.[2]

3. Dose response and time course of the action of methoxamine and carbamylcholine. Although both methoxamine and carbamylcholine inhibit the *beta* adrenergic stimulation of cyclic AMP formation, the kinetic characteristics of this inhibition differ considerably. Carbamylcholine was more potent than methoxamine, and its action did not increase appreciably at concentrations greater than 5–10 μM. Methoxamine exhibited significant inhibition at concentrations greater than 0.5 μM, and its inhibition increased over the whole range of concentrations tested (up to 250 μM). At concentrations greater than 50 μM, its action was not fully blocked by either phentolamine or phenoxybenzamine (used at maximal concentrations specific for *alpha* adrenergic receptor). Thus the action of methoxamine at higher concentrations cannot be attributed exclusively to its *alpha* adrenergic properties. It is conceivable that methoxamine exerts its effects at very high concentrations by competing with (−)-isoproterenol for the binding to the *beta* adrenergic receptor.

Carbamylcholine displayed a fast inhibition and produced almost its maximal effect when added simultaneously with (−)-isoproterenol. We did not observe the biphasic time-course of inhibition reported by Harper and Brooker (4), who used norepinephrine as a *beta* adrenergic agonist. Methoxamine required more than 8 min to produce its maximal effect under similar conditions. It did not significantly reduce the cyclic AMP concentration when added together with (−)-isoproterenol without preincubation.

4. The role of extracellular Ca^{++} in alpha adrenergic and cholinergic effects. It is generally accepted that the *alpha* adrenergic and cholinergic effects in the parotid (as well as in many other tissues) are mediated by influx of extracellular Ca^{++}. The action of *alpha* adrenergic and cholinergic agonists can be resolved into primary events leading to increased cell membrane permeability to Ca^{++} and other biochemical changes (e.g., increased incorporation of $^{32}P_i$ into phosphatidylinositol), and a second class of events entirely dependent on extracellular Ca^{++}, mimicked by the divalent cation ionophore in the presence of Ca^{++} and satisfactorily explained in terms of increasing the concentrations of cytosolic Ca^{++} (e.g, K^+ efflux and vacuole formation in rat parotid). The action of methoxamine belongs clearly to the second class of events. Its action is entirely dependent on extracellular Ca^{++} and does not produce any effect in the presence of A23187 and Ca^{++}. Moreover, the dose-response curve to (−)-isoproterenol in the presence of A23187 and Ca^{++} resembles the curve obtained in the presence of methoxamine.

Carbamylcholine, on the other hand, acts, at least in part, as a primary effector; i.e., it exerts its full action in the absence of extracellular Ca^{++}, and also in the presence of A23187 and Ca^{++}. Under the last set of conditions, the cell is flooded with Ca^{++} and maximal K^+ efflux is obtained. This will argue against carbamylcholine requiring low extracellular Ca^{++} or using intracellular Ca^{++} for its effects. One cannot rigorously exclude the existence of an intracellular Ca^{++} pool that is inaccessible to A23187 and serves as a target for cholinergic agonists. We would prefer, however, not to invoke this type of argument until the existence of the hypothetical pool is demonstrated.

The proof of a Ca^{++}-dependent component in carbamylcholine action is complicated by a frequent inhibition of cyclic AMP accumulation elicited by (−)-isoproterenol in the absence of exogenous Ca^{++} [2], and the invariably large inhibition in the presence of A23187 and Ca^{++}. Since it has been demonstrated that cholinergic acti-

[2] Oron & Lamer, unpublished results.

vation leads to Ca^{++} uptake in rat parotid cells (24–26), we can assume carbamylcholine exerts its action on cyclic AMP regulation also via a Ca^{++}-dependent mechanism. The report by Harper and Brooker (4) describing a fast Ca^{++}-independent cholinergic inhibition followed by a slower Ca^{++}-dependent inhibition bears out this assumption.

5. The patterns of cholinergic and alpha adrenergic inhibition. The non-hyperbolicity of the dose-response curve to (−)-isoproterenol makes formal analysis of inhibition data difficult. It appears that both carbamylcholine and methoxamine cause a limited and apparently competitive inhibition at low concentrations of (−)-isoproterenol. The data presented by Butcher *et al.* (22) and by us support the apparently competitive mode of *alpha* adrenergic inhibition in the case of methoxamine and mixed-adrenergic agonists. At higher concentrations of (−)-isoproterenol the inhibition increased and appeared to be noncompetitive. The inhibition caused by A23187 + Ca^{++} appears to be similar.

6. The target of cholinergic and alpha adrenergic inhibition. The reduction in cyclic AMP concentrations observed with both *alpha* adrenergic and cholinergic stimulation could be effected by the inhibition of adenylate cyclase, stimulation of cyclic phosphodiesterase, or both. In order to determine which of the possibilities is correct, we assayed the action of carbamylcholine and methoxamine in the presence of cyclic AMP phosphodiesterase inhibitors. Both agonists exert their action under these conditions, albeit, the inhibition appears relatively smaller than in the absence of phosphodiesterase inhibitors. Only the complete absence of inhibition, or exactly the same degree of inhibition, will provide an unequivocal proof for the involvement of phosphodiesterase, or the lack of it.

The rate of disappearance of cyclic AMP in the presence of carbamylcholine was not significantly greater than in the control system. Because of a slow action of methoxamine and fast rate of cyclic AMP disappearance, the same experiment could not be performed with methoxamine.

The results appear to favor adenylate cyclase as a target for cholinergic and *alpha* adrenergic action. We have recently observed a stable inhibition of adenylate cyclase in membranes isolated from slices preexposed to carbamylcholine. This finding supports the conclusion that adenylate cyclase is the target enzyme. We are currently studying the characteristics of this inhibition in an attempt to elucidate its molecular mechanism.

7. The difference between cholinergic and alpha adrenergic inhibition: A possible mechanism. Although the actions of cholinergic and *alpha* adrenergic agonists appear to be identical when K^+ efflux, phospholipid effect and cell morphology are concerned, there are fundamental differences in their cyclic AMP-regulation capacity. For the sake of clarity the differences have been compiled into a table:

Parameter	*Alpha* adrenergic effect	Cholinergic effect
Dependence on extracellular Ca^{++}	Yes	No
Action in the presence of increased intracellular Ca^{++}	No	Yes
Kinetics of action	Slow	Fast

The data presented here warrant an assumption of different mechanisms of action for carbamylcholine and methoxamine. The lack of additivity of the effects of both agents is explained by the fact that carbamylcholine exhibits both Ca^{++}-dependent and Ca^{++}-independent activity. One would expect that under identical conditions carbamylcholine will produce greater inhibition than methoxamine. This is generally the case, but interpretation is complicated by the lack of saturation of the methoxamine dose-response curve.

Recently a cell-free effect was reported on the adenylate cyclase of the heart sarcolemma (27). In this system cholinergic agonists appear to affect the guanine-nucleotide regulatory site. We are currently investigating the same parameters in the rat parotid.

The Ca^{++}-dependent action of carbamylcholine and methoxamine could be explained in several possible ways:

A. A direct effect of increased Ca^{++} on

adenylate cyclase.

B. A direct effect of increased Ca^{++} on phosphodiesterase and phosphodiesterase-modulator protein.
C. Ca^{++} competition with Mg^{++} to form the inhibitory substrate CaATP.
D. A sharp drop in ATP concentrations due to Ca^{++} influx and K^+ efflux.
E. An undetermined secondary event connected with Ca^{++} influx.

Although none of the above can be excluded rigorously at this stage, the slow onset of Ca^{++}-dependent inhibition and the relatively incomplete reversibility after prolonged incubation with blockers favor the last possibility. One cannot exclude a possible release of a second substance (e.g., peptide hormone), which might mediate the inhibitory effects.

8. Physiological significance. The major role of the *beta* adrenergic receptor in the rat parotid is to control the secretion of exportable protein stored in secretory granules of the parotid acinar cells. Cholinergic and *alpha* adrenergic receptors control independently K^+ efflux accompanying water secretion. These events were integrated into an overall scheme by Schramm and Selinger (1). Sufficient evidence has accumulated to justify the incorporation of the *alpha* adrenergic and cholinergic control of cyclic AMP production into this scheme.

It is well documented that a small rise in cyclic AMP concentration will maximally stimulate the secretion of exportable proteins. The large increases in cyclic AMP concentration due to the stimulation of the *beta* adrenergic receptor appear to be physiologically unnecessary. The activation of cholinergic receptors combined with the simultaneous activation of the *alpha* adrenergic receptor by the natural mixed adrenergic agonist norepinephrine will result in levels of cyclic AMP that are much lower but still sufficient to account for maximal rate of secretion. Moreover, both *alpha* adrenergic and cholinergic agonists appear to exert their action on cyclic AMP levels at concentrations well below those which cause measurable K^+ efflux. It is, therefore, conceivable that they might control cyclic AMP levels before they trigger K^+ and water efflux. The parotid provides a model system for inter-receptor modulation and also a system for the biochemical analysis of the molecular mechanism of action of *alpha* adrenergic and cholinergic receptor-mediated events.

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