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Refractoriness to Muscarinic and Adrenergic Agonists in the Rat Parotid: Responses of Adenosine and Guanosine Cyclic 3',5'-Monophosphates

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SUMMARY

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Cyclic 3',5'-AMP and cyclic 3',5'-GMP elevations in response to various receptor agonists are reduced upon repeated stimulation in rat parotid. The concentration of cyclic AMP in rat parotid is elevated upon stimulation with either *beta* adrenergic or muscarinic receptor agonists, although the cholinergic receptor agonist carbachol produces only 1% of the maximal response to the *beta* adrenergic receptor agonist isoproterenol. In both cases the cyclic AMP elevation is transient, and readdition of the agent used to produce the stimulation does not lead to additional cyclic AMP accumulation, showing that the tissue has become refractory. Removal of extracellular calcium does not prevent elevations of cyclic AMP or refractoriness after stimulation with either norepinephrine or carbachol. The simultaneous addition of carbachol and norepinephrine in the presence and absence of calcium does not produce as great an elevation of cyclic AMP as can norepinephrine alone. Carbachol also produces a time- and calcium-dependent, nonspecific refractoriness to norepinephrine-mediated cyclic AMP accumulation. Cyclic AMP accumulation in the parotid therefore shows receptor-nonspecific refractoriness, since stimulation by any agent capable of elevating cyclic AMP leads to cyclic AMP accumulation refractory to all agonists. Cyclic GMP refractoriness can also be observed in the parotid. Carbachol and norepinephrine are able to stimulate the accumulation of cyclic GMP through muscarinic and *alpha* adrenergic receptors, respectively, while sodium azide stimulates its accumulation through a different mechanism. Extracellular calcium is required for carbachol or norepinephrine to stimulate cyclic GMP accumulation, although azide is effective in its absence. Unlike the interaction of carbachol and norepinephrine toward cyclic AMP concentration, the simultaneous addition of these drugs produces a cyclic GMP accumulation no different from the maximal response to either agent alone. Both carbachol

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and norepinephrine produce refractoriness to readdition of the same agent, but neither is capable of producing that state in response to drugs acting through receptors not originally involved. Extracellular calcium is not required for the induction of cyclic GMP refractoriness, and thus refractoriness is not triggered by the accumulation of cyclic GMP. Cyclic GMP refractoriness is receptor-specific in the same tissue in which cyclic AMP elevation exhibits a receptor-nonspecific refractory pattern.

INTRODUCTION

The intracellular control of cell surface hormone-receptor interactions is not well understood, although it may be an important physiological process by which hormone responses can be regulated on an acute or long-term basis. Many hormones that interact with membrane receptors are able to stimulate the accumulation of cyclic 3′,5′-AMP or cyclic 3′,5′-GMP. Cyclic nucleotide elevation is thought to be an early step in hormone action, and thus information about hormone responsiveness and cyclic nucleotide kinetics should shed light upon the regulation of hormone action. In nearly every tissue, once cyclic AMP is elevated by a hormone, it quickly reaches a peak concentration en route to a return toward its basal level. Following the first description by Kakiuchi and Rall (1) of what has been called cyclic AMP refractoriness, it has often been noted that this inexorable decline of cyclic AMP concentration cannot be altered by a fresh application of the originally effective hormone. This refractory phenomenon may be specific to the hormone used, so that agents capable of stimulating the accumulation of cyclic AMP through other, distinct receptors are fully active in tissue refractory to a first hormone (1–6). This is called receptor-specific refractoriness.[3] On the other hand, stimulation by one hormone may cause the tissue not to respond to any normally effective agent (7, 8); this is termed receptor-nonspecific refractoriness.

While hormones are known to cause a transient elevation of cyclic GMP in several tissues, only recently have any reports of cyclic GMP refractoriness appeared. In those studies the *beta* adrenergically stimulated cyclic GMP accumulation in cultured C-6 cells was shown to become refractory after several hours of isoproterenol treatment (9, 10). An understanding of the factors that regulate parotid cyclic GMP concentrations could lead to further knowledge of the possible roles of cyclic GMP in this tissue. The present study was also undertaken to explore the possibility of the development of refractoriness of both cyclic nucleotides in one tissue.

MATERIALS AND METHODS

The cyclic AMP antiserum was prepared by Dr. Wes Terasaki in our laboratory, using goats to produce serum against 2′*O*-succinyl cyclic AMP coupled to human serum albumin. The cyclic GMP antiserum was a generous gift from Dr. Ferid Murad (11). We prepared the radioiodinated cyclic nucleotides with succinyl cyclic nucleotide tyrosine methyl esters obtained from Sigma and $Na^{125}I$ from New England Nuclear, using the method of Hunter and Greenwood (12) as modified (13). *l*-Propranolol was a kind gift from Dr. T. J. Franklin of ICI, Ltd.; carbachol (carbamylcholine chloride), *l*-isoproterenol HCl, *l*-norepinephrine HCl, atropine sulfate, sodium azide, and bovine serum albumin (fraction V) were from Sigma. Other compounds were reagent grade or

[3] The refractoriness of cyclic nucleotide accumulation to restimulation with a hormone that acts at the same receptor as does the hormone used as the initial stimulus is called receptor-specific refractoriness (specific refractoriness). If restimulation with a hormone that normally elevates the cyclic nucleotide through interaction with any receptor exhibits cyclic nucleotide refractoriness after initial stimulation with any one such agent, the tissue is said to exhibit receptor-nonspecific refractoriness (nonspecific refractoriness). If cyclic AMP accumulation is used as the measure of refractoriness, cyclic AMP refractoriness is said to exist. Similarly, cyclic GMP refractoriness refers to the inability of a hormone to elevate the cyclic GMP concentration after prior stimulation.

better. Deionized water was glass-distilled prior to use.

Preparation of tissue. Male Wistar rats (Hilltop Lab Animals, Scottdale, Pa., 150–350 g) were stunned by a blow to the head and exsanguinated. Parotid glands were removed and rapidly placed in Krebs-Ringer-bicarbonate buffer (pH 7.3 at 37°) containing 6.1 mM D-glucose and 6.1 mM β-hydroxybutyrate with the following ionic composition: Na^+, 143 mM; K^+, 5.9 mM; Mg^{2+}, 1.2 mM; Ca^{2+}, 2.5 mM; Cl^-, 127.7 mM; SO_4^{2-}, 1.2 mM; PO_4^{3-}, 1.2 mM; HCO_3^-, 25 mM. The buffer had previously been gassed with 95% O_2–5% CO_2 and was kept under an atmosphere of that gas. Once removed, parotids were dissected free of extraneous macroscopic tissue, cut into approximately 1-mm cubes, and initially incubated in fresh buffer for 30–60 min. Except for short periods during dissection, the tissue was maintained at 37°. In experiments using calcium-free buffer, the tissue was treated normally until introduction of that buffer at the initial incubation step. At the end of the initial incubation period aliquots of parotid pieces were placed in 17 × 100 mm polypropylene test tubes containing 1.0 ml of either normal or calcium-free buffer. After each manipulation of tissue, or at least every hour, the vessels were regassed and stoppered. Continuous gassing with 95% O_2–5% CO_2 was equally effective in preserving tissue cyclic nucleotide response; adequate oxygenation was maintained by either method. Each tissue incubation was terminated by the addition of 50 μl of 100% (w/v) trichloracetic acid, followed by homogenization for 15 sec with a Polytron PT-10 instrument at setting 7. The homogenates were centrifuged to remove the precipitated protein, and the supernatant solutions were then decanted, extracted three times with 5 volumes of water-saturated diethyl ether, and heated at 50° for 25 min to remove the residual ether. The pellets were dissolved in 2 ml of 0.5 N NaOH and assayed for protein by the method of Lowry *et al.* (14), adapted to a continuous flow system, using bovine serum albumin as the standard.

Radioimmunoassay procedure. Cyclic AMP and cyclic GMP radioimmunoassays (15) were performed using acetylated samples, essentially as previously described (13). Briefly, 500-μl aliquots of the ether-extracted samples were acetylated with 10 μl of triethylamine and 5 μl of acetic anhydride. The acetylated samples were used to assay both cyclic AMP and cyclic GMP. In each case aliquots of acetylated samples were incubated with an antibody specific for the cyclic nucleotide and the radioiodotyrosine methyl ester derivative of the appropriate succinyl cyclic nucleotide. The incubation buffer was 50 mM sodium acetate, pH 4.75, containing 10 mg/ml of bovine serum albumin. Cyclic GMP assay mixtures were incubated overnight at 4° before removal of the free radioactive tracer by absorption to charcoal. Cyclic AMP assays were performed with a Gammaflow automated radioimmunoassay system (16). Because of the high sensitivity and selectivity of assay afforded by the acetylation procedure, no purification of cyclic nucleotides was necessary.

Expression of results. Each value of the experimental results represents the mean value of the indicated number of distinct experiments, each experimental value being the mean of two to five separate incubation tubes of parotid pieces obtained from one pool of tissues made from the glands of two to four rats. Cyclic AMP and cyclic GMP are expressed in picomoles per milligram of protein or as "fold above" the basal level, which was calculated for each experiment by dividing the mean post-treatment cyclic nucleotide concentration by the mean basal one, i.e., ([cyclic nucleotide] after stimulus)/(basal [cyclic nucleotide]), where the basal value is taken as 1.0-fold. The means from each experiment were then averaged to yield the reported mean ± standard error. The mean basal cyclic AMP concentration was 3.25 ± 0.14 pmoles/mg of protein (101 experiments), and the mean basal concentration of cyclic GMP was 71.1 ± 8.9 fmoles/mg of protein (36 experiments). The coefficient of variation for cyclic AMP values is similar when expressed either as a concentration or as fold above basal, but because of the large variability in the basal cyclic GMP concentration (the coef-

ficient of variation for 36 basal values was 74.1%) fold-above-basal expressions for this cyclic nucleotide showed less variation and made easier the comparisons between various treatments. There was less variability among replicates within any experiment. The coefficients of variation of cyclic AMP concentrations were usually less than 10%, while for cyclic GMP they averaged less than 20%. Student's *t*-test was used for the determination of statistical significance. Comparisons of treatments with each corresponding control were made using the paired *t*-test for cyclic nucleotide concentrations, while the unpaired *t*-test was used on fold-above-basal values.

RESULTS

Cyclic AMP is rapidly elevated in the rat parotid upon stimulation with 50 μM *l*-isoproterenol, the maximal dose (Fig. 1). The peak cyclic AMP concentration is reached at 1–2 min, at which time a rapid decline begins. By 10 min the rate of decline slows, so that the return toward the basal concentration proceeds over the next 3 hr without returning to that level. The extended time without a return to the basal concentration, while accentuated by the use of the maximal isoproterenol concentration, also occurs after stimulation with much lower concentrations. At 100 nM, isoproterenol stimulates cyclic AMP to a peak of only 20% of the maximally stimulated concentration, yet after 1 hr cyclic AMP is still elevated to twice the basal concentration (data not shown).

If isoproterenol (50 μM) is readded to parotid pieces 6, 28, or 178 min after a first addition of the drug, the cyclic AMP concentration is not significantly elevated from the level in tissue without a catecholamine readdition (Fig. 2). This failure to elicit a second response to isoproterenol is statistically significant, since the cyclic AMP concentration after isoproterenol readdition is lower than that achieved with a normal 2-min incubation, even without allowing for the elevated baseline of cyclic AMP obtained after a first incubation. Although in this and other refractoriness experiments the second drug addition was terminated after 2 min, at no time (1–30 min) after the second addition is cyclic AMP stimulated above the existing concentration.

Carbachol is also able to stimulate the accumulation of cyclic AMP, although with quite low efficacy compared with *beta* adrenergic stimulation (Table 1); 20 and 200 μM concentrations of carbachol are

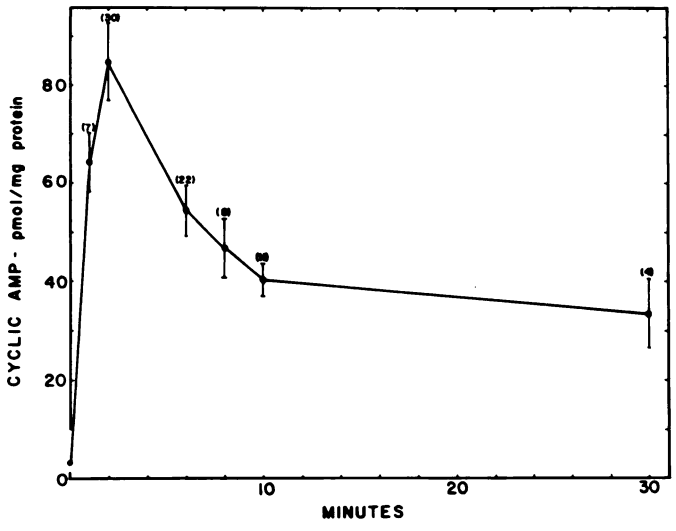

FIG. 1. *Isoproterenol time course*

Cyclic AMP accumulation in rat parotid pieces (picomoles per milligram of protein) is shown as a function of time after the addition of 50 μM *l*-isoproterenol. The number of individual experiments performed at each point is given in parentheses. Bars indicate standard errors of the mean.

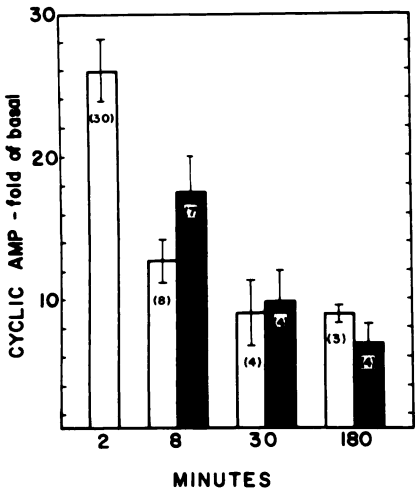

FIG. 2. *Cyclic AMP refractoriness*

Cyclic AMP accumulation in rat parotid pieces (mean multiple or "fold above" basal level ± standard error) at various times after the addition of 50 μM *l*-isoproterenol is shown as the open bars. The solid bars show the cyclic AMP accumulation attained upon a second addition of 50 μM isoproterenol to the same incubation tubes for the final 2 min of isoproterenol stimulation, whose total duration is indicated below the bars. The number of individual experiments performed at each point is shown in parentheses.

equally able to elevate the cyclic AMP concentration at 2 min. This effect is potentiated by the addition of 1% 1-propanol, which has little effect by itself. Potentiation of the accumulation of cyclic AMP is also seen upon *beta* adrenergic stimulation in the presence of propanol (data not shown). When so potentiated, 20 μM carbachol is able to increase the cyclic AMP content by 60% over the level produced by propanol alone. This stimulation can be blocked completely with 20 μM atropine ($p < 0.005$ vs. carbachol with propanol), but is unaffected by 10 μM *l*-propranolol, indicating that carbachol acts not through release of a small amount of endogenous catecholamine but directly on a muscarinic receptor. The effect of propanol upon carbachol or norepinephrine stimulation is rather specific, since it does not lead to any potentiation of their ability to elevate cyclic GMP (data not shown). In the absence of extracellular calcium, carbachol is fully able to cause an elevation of cyclic AMP to a concentration not significantly different from that produced by carbachol in the presence of readded calcium (Table

TABLE 1

Effect of carbachol on cyclic AMP

Parotid tissue was treated with the stated compounds for the indicated lengths of time. The atropine concentration was 20 μM; *l*-propranolol, 10 μM; and 1-propanol, 1% (134 mM). Results from tissue incubated in calcium-free buffer are denoted by "Ca-free." $CaCl_2$ was added with carbachol (CCh) for the 2-min stimulation at a final concentration of 2.5 mM. Cyclic AMP is presented as mean multiple or "fold above" the basal level (mean ± standard error), with the number of individual experiments performed for each data point expressed in the third column. Atropine blocks the effects of carbachol with propanol ($p < 0.005$).

Treatment	Time	*n*	Cyclic AMP
	min		*-fold/basal*
20 μM CCh	2	29	1.26 ± 0.03[a]
20 μM CCh	60	7	0.87 ± 0.06
20 μM CCh + atropine	2	3	0.94 ± 0.11
20 μM CCh + propanol	2	6	1.82 ± 0.18[b]
20 μM CCh + propanol + atropine	2	5	1.09 ± 0.02
20 μM CCh + propanol + propranolol	2	5	1.64 ± 0.10[a]
20 μM CCh (Ca-free)	2	5	1.10 ± 0.03[c]
20 μM CCh + $CaCl_2$ (Ca-free)	2	5	1.20 ± 0.07[c]
200 μM CCh	2	13	1.32 ± 0.05[a]
200 μM CCh	60	5	1.03 ± 0.18
Propanol	2	6	1.17 ± 0.07

[a] $p < 0.001$ vs. control.
[b] $p < 0.01$ vs. control.
[c] $p < 0.05$ vs. control.

TABLE 2

Cyclic nucleotide refractoriness

The accumulation of cyclic AMP and cyclic GMP (as "fold above" basal, mean ± standard error) after various treatments is shown. The first treatment was given for 60 min, and the second treatment for the final 2 min of that incubation; if no treatment is listed, nothing was added at the time in question. The number of individual experiments averaged to obtain each reported value is given in the column immediately preceding that set of values. The abbreviations used are: NE, *l*-norepinephrine HCl; CCh, carbachol chloride.

First treatment (60 min)	Second treatment (2 min)	*n*	Cyclic AMP	*n*	Cyclic GMP
			× *basal*		*-fold/basal*
	25 μM NE	29	21.2 ± 2.1[a]	16	2.20 ± 0.18[a]
	20 μM CCh	29	1.26 ± 0.03[a]	18	2.66 ± 0.16[a]
	200 μM CCh	13	1.32 ± 0.05[a]	8	2.83 ± 0.21[b]
	20 μM CCh + 20 μM atropine	3	0.94 ± 0.11	3	0.98 ± 0.04
	20 μM CCh + 25 μM NE	16	14.1 ± 1.3[a]	6	2.60 ± 0.32[b]
	200 μM CCh + 25 μM NE	4	9.6 ± 1.7[a]		
25 μM NE		8	6.7 ± 0.9[b]	7	1.25 ± 0.07[c]
20 μM CCh		7	0.87 ± 0.06	9	1.27 ± 0.06[b]
200 μM CCh		5	1.03 ± 0.18	5	1.68 ± 0.22[c]
25 μM NE	25 μM NE	10	6.5 ± 0.7[d]	9	1.55 ± 0.19[e]
25 μM NE	20 μM CCh			3	3.01 ± 0.64[f]
20 μM CCh	20 μM CCh	6	0.92 ± 0.04[d]	8	1.26 ± 0.11[d]
20 μM CCh	25 μM NE	13	8.8 ± 0.5[g]	7	1.77 ± 0.16[f]
200 μM CCh	200 μM CCh	6	0.94 ± 0.07[d]	6	1.33 ± 0.17[d]
200 μM CCh	25 μM NE	5	5.2 ± 0.6[g]	3	2.14 ± 0.09[f]
20 μM CCh + 20 μM atropine	25 μM NE	5	25.3 ± 5.0[f]	5	1.83 ± 0.09[f]

[a] $p < 0.001$ vs. basal.
[b] $p < 0.01$ vs. basal.
[c] $p < 0.05$ vs. basal.
[d] $p < 0.001$ for specific refractoriness (i.e., 2-min point is higher than 60- plus 2-min elevation).
[e] $p < 0.01$ for specific refractoriness.
[f] $p < 0.02$ for lack of nonspecific refractoriness (or significance of elevation by a heterologous agonist).
[g] $p < 0.02$ for nonspecific refractoriness.

1). This is contrary to the prevention of cyclic GMP elevation by the removal of calcium (see Table 3).

Readdition of either 20 or 200 μM carbachol 58 min after a first stimulation with that agent does not produce any increase in cyclic AMP concentration (Table 2). Thus cyclic AMP refractoriness occurs with cholinergic stimulation as well as with *beta* adrenergic agonists such as isoproterenol and norepinephrine. The exclusion of calcium from the incubation buffer does not prevent carbachol-induced cyclic AMP refractoriness, and readdition of calcium along with carbachol to refractory tissue yields no stimulation of refractory tissue (Table 3).

Although carbachol itself produces only a small elevation of cyclic AMP, its effect on the norepinephrine-induced stimulation of cyclic AMP is quite dramatic. Carbachol (20 μM) inhibits the normal 2-min cyclic AMP response to 25 μM norepinephrine (Fig. 3): the simultaneous addition of carbachol with norepinephrine leads to 36% inhibition of the response to norepinephrine alone ($p < 0.001$). Butcher *et al.* (17) have previously reported the occurrence of this immediate effect. It is probably distinct from a second type of refractoriness in this tissue, since neither time nor extracellular calcium is necessary for its development. The time-dependent development of nonspecific refractoriness is

TABLE 3

Lack of effect of calcium removal on refractoriness

Tissue incubated in the presence or absence of extracellular calcium was stimulated by 20 μM carbachol or 25 μM *l*-norepinephrine. Each experiment was performed in quadruplicate, using tissue pooled from four rats, and is representative of experiments repeated at least three times. Other conditions were the same as in Table 2. The abbreviations used are: CCh, carbachol chloride; NE, norepinephrine.

Expt.	First treatment (60 min)	Second treatment (2 min)	Buffer calcium	Cyclic AMP	Cyclic GMP
				pmoles/mg	*fmoles/mg*
I		Water	+	1.65 ± 0.14	24.9 ± 1.9
		CCh	+	2.46 ± 0.29	76.2 ± 7.6
	CCh		+	1.38 ± 0.04	30.7 ± 2.3
	CCh	CCh	+	1.58 ± 0.09	33.9 ± 8.1
		Water	−	1.70 ± 0.05	21.5 ± 0.8
		$CaCl_2$	−	1.76 ± 0.04	28.4 ± 1.7
		CCh	−	1.87 ± 0.08	21.7 ± 0.2
		CCh + $CaCl_2$	−	1.94 ± 0.08	68.8 ± 14.6
	CCh	CCh + $CaCl_2$	−	1.63 ± 0.07	32.6 ± 5.7
II		Water	+	1.52 ± 0.02	38.9 ± 1.3
		NE	+	24.9 ± 0.48	87.0 ± 31.2
	NE		+	10.0 ± 0.85	40.1 ± 8.9
	NE	NE	+	8.1 ± 0.63	33.0 ± 5.2
		Water	−	1.55 ± 0.13	32.8 ± 3.2
		$CaCl_2$	−	1.50 ± 0.07	51.5 ± 9.2
		NE	−	16.0 ± 0.96	35.1 ± 1.7
		NE + $CaCl_2$	−	26.7 ± 2.8	92.7 ± 10.0
	NE	NE + $CaCl_2$	−	7.6 ± 0.31	42.2 ± 4.8

apparent from Fig. 3. When carbachol is added 2–58 min prior to a norepinephrine challenge, inhibition of the normal response to norepinephrine is significantly greater than when the two drugs are added simultaneously. A series of experiments similar to those described in Fig. 3 was performed, with half the parotid pieces incubated in calcium-free buffer. The results of a representative experiment are shown in Fig. 4. The absence of calcium has no effect upon the immediate inhibition of a 2-min norepinephrine stimulation of cyclic AMP by carbachol, although the further time-dependent inhibition of cyclic AMP elevation cannot occur without extracellular calcium. Calcium does not have to be present throughout the carbachol incubation, since adding it at the same time as the norepinephrine challenge allows carbachol to exert its normal inhibitory effect. Thus there is less cyclic AMP accumulation with addition of norepinephrine plus $CaCl_2$ after 28 min of carbachol than if norepinephrine alone is used as the second addition.

The inhibitory effect of carbachol is apparent as a change in norepinephrine efficacy, since the addition of 300 μM norepinephrine after 58 min of carbachol is no more effective than is 25 μM norepinephrine (data not shown). The cyclic AMP refractoriness produced by carbachol is entirely prevented by atropine, as are its effects on the elevation of cyclic AMP and cyclic GMP (Table 2).

Figure 5 details the dose-response relationships for the ability of isoproterenol to stimulate cyclic AMP accumulation, measured at 2 min, and to reduce the norepinephrine response 58 min later. The conditions were identical with those used to test the effect of carbachol after 58 min of stimulation with carbachol, but whereas a 25% ± 4% increase in cyclic AMP concentration after carbachol leads to a 43% ± 5% (n = 9) decrease in the normal 2-min response to carbachol plus norepinephrine (and a decrease of 63% ± 4% in the response to norepinephrine alone), a cyclic AMP elevation of 125% ± 21% by 10 nM isoproterenol is needed to produce a 27%

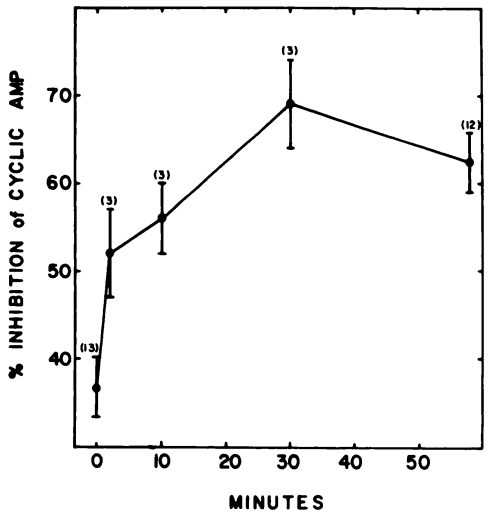

FIG. 3. *Receptor-nonspecific cyclic AMP refractoriness*

Inhibition of the normal 2-min cyclic AMP elevation above basal after 25 μM *l*-norepinephrine is shown as a function of the duration of prior stimulation with 20 μM carbachol. The abscissa shows the length of carbachol stimulation prior to the addition of norepinephrine to the parotid slices for the final 2 min of the incubation. Norepinephrine alone produces a mean cyclic AMP concentration of 57.9 ± 7.3 pmoles/mg of protein (n = 23) at 2 min. Percentage inhibition was calculated for each experiment. The numbers in parentheses indicate the number of individual experiments averaged to yield the reported means ± standard errors.

± 6% specific refractoriness of the norepinephrine response. Therefore either cyclic AMP elevations are unrelated to the induction of cyclic AMP refractoriness or the effect of carbachol is to produce a more efficient cyclic AMP refractoriness coupling, or a refractoriness factor in addition to cyclic AMP. It is also apparent from Fig. 5 that isoproterenol is about one order of magnitude more potent in making the tissue refractory than in causing the acute elevation of cyclic AMP.

Stimulation of parotid pieces by norepinephrine or carbachol also leads to the elevation of cyclic GMP; in this case the drugs act through *alpha* adrenergic and muscarinic receptors, respectively. Unlike the situation seen with the elevation of cyclic AMP, these drugs are equally able to elevate cyclic GMP (Table 2). Furthermore, elevation of cyclic GMP by these agents is completely prevented by the removal of extracellular calcium (Table 3). Cyclic GMP refractoriness occurs with both *alpha* adrenergic and muscarinic agonists. When carbachol or norepinephrine is added to parotid pieces at zero time, followed by readdition of the same drug 58 min later, the tissue is found to have become refractory. No elevation of cyclic GMP above the expected 60-min post-stimulation concentration can be obtained 1–30 min after a second stimulation with that agent. Data are shown for the 2-min (maximal) stimulation in Table 2. While this holds true for both classes of neuroreceptor agonists, no nonspecific refractoriness between the two is seen. Thus carbachol does not produce refractoriness to the norepinephrine effect on cyclic GMP accumulation, although it has profound effects upon the ability of norepinephrine to elevate cyclic AMP. When the two drugs

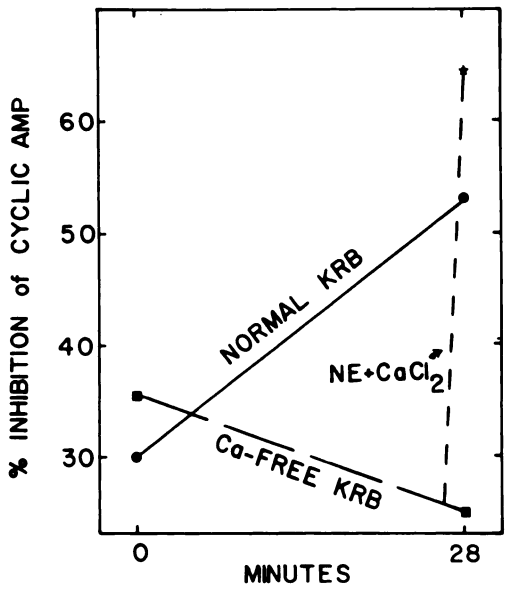

FIG. 4. *Effect of calcium on nonspecific cyclic AMP refractoriness*

Conditions were the same as described for Fig. 3, except that half the tissue was incubated in calcium-free Krebs-Ringer-bicarbonate (KRB; - - -). In this representative experiment, 28 min after the addition of 20 μM carbachol, a 2-min challenge with either 25 μM norepinephrine (NE; ● and ■) or norepinephrine plus 2.5 mM $CaCl_2$ (★) was performed. Tissue was obtained from a common pool made of the glands of four rats; duplicate incubations were performed, with a mean coefficient of variation of 6.6% among the 16 data pairs.

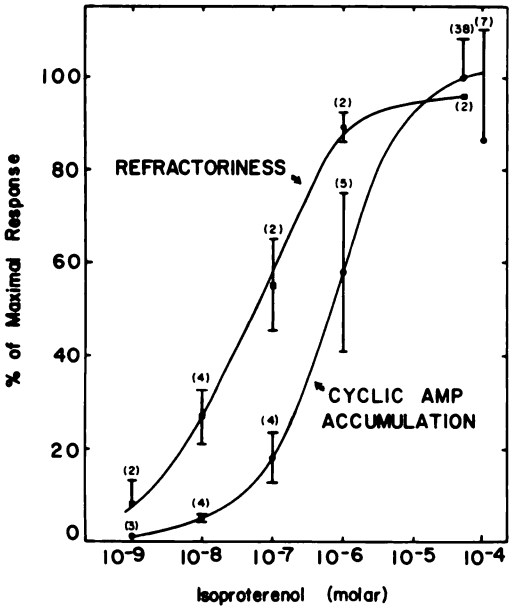

FIG. 5. *Dose-response relationships for cyclic AMP accumulation and refractoriness*

●, cyclic AMP concentration obtained after 2 min with the indicated concentration of isoproterenol, labeled "cyclic AMP accumulation"; ■, refractoriness of the normal 2-min response to 25 μM *l*-norepinephrine produced by various concentrations of isoproterenol added 58 min previously, labeled "refractoriness." The ordinate is calibrated in percentage of the maximal response. Cyclic AMP accumulation ranged from 1.00-times the individual experimental basal concentration (i.e., zero) to 28.1-fold, which is the mean cyclic AMP concentration attained with 50 μM *l*-isoproterenol. Refractoriness ranged from zero (allowing cyclic AMP to accumulate to the concentration achieved with the 25 μM norepinephrine control in each experiment) to complete refractoriness toward the ability of norepinephrine to elevate cyclic AMP. The number of individual experiments performed at each point is shown in parentheses. Bars indicate standard errors of the mean.

are added simultaneously, the maximal cyclic GMP accumulation is no different from that produced by either agent alone (Table 2), again in contrast to the immediate inhibitory effect of carbachol on cyclic AMP accumulation stimulated by norepinephrine.

The specific cyclic GMP refractoriness that develops after stimulation with either carbachol or norepinephrine does not seem to be related to the acute accumulation of intracellular cyclic GMP. Cyclic GMP refractoriness is fully expressed even when the 58-min first incubation proceeds in the absence of extracellular calcium (Table 3), since with readdition of calcium the refractory tissue is unable to respond. Calcium replacement for a 2-min drug stimulation in the absence of refractoriness allows full expression of cyclic GMP accumulation (Table 3).

Sodium azide is able to stimulate cyclic GMP accumulation in the parotid via a mechanism distinct from adrenergic and cholinergic receptors. Azide-stimulated cyclic GMP elevation is unaffected by atropine, phentolamine, propranolol, or the elimination of extracellular calcium, conditions that block cyclic GMP stimulation in parotid by all other known effective agents (data not shown). Carbachol-treated tissue does not become refractory to azide (Table 4), again showing a lack of nonspecific cyclic GMP refractoriness. Azide has no effect on cyclic AMP (data not shown).

DISCUSSION

The rat parotid has previously been shown to respond in various ways to neurotransmitters involving three receptor types. Stimulation of *beta* adrenergic receptors produces a variety of effects. The

TABLE 4

Lack of refractoriness to sodium azide after carbachol

Sodium azide (300 μM) was added for the final 10 min of a 60-min incubation; in some experiments 200 μM carbachol was added at the start of the experiment. Cyclic GMP values are the means ± standard errors for the number of experiments presented in the preceding column. Azide treatment elevates cyclic GMP above the basal level ($p < 0.0001$); the same level of confidence was obtained for the lack of nonspecific refractoriness (200 μM carbachol for 60 min with azide for the final 10 min vs. carbachol alone for 60 min).

First treatment (60 min)	Second treatment (10 min)	*n*	Cyclic GMP
			-fold/basal
	Azide	7	2.46 ± 0.23
Carbachol		5	1.68 ± 0.22
Carbachol	Azide	6	2.76 ± 0.27

acinar cells are essentially depleted of secretory granules within 90 min of maximal stimulation (18). This effect has been correlated with a rapid, large increase in cyclic AMP concentration (19). Recently it has been reported that a *beta* adrenergic interaction is able to stimulate the release of potassium and to elevate cyclic GMP (20), although with quite low efficacy compared with the stimulation of these effects by *alpha* adrenergic or muscarinic agonists (17, 20–22). Cholinergic and *alpha* adrenergic stimulation also leads to the release of secretory granules, although maximal stimulation with these agents is only one-quarter of that possible with isoproterenol (17, 23, 24). Others have stated that carbachol alone has no effect upon cyclic AMP concentration in the rat parotid (17, 23). Negative results for the rabbit parotid have also been reported (25). That we have found such an effect can be explained by several reasons. A large number of experiments were performed at two concentrations of carbachol, with and without extracellular calcium, and a potentiating agent was discovered. Each of the three treatments produced significant elevations of parotid cyclic AMP, while the effects of atropine and *l*-propranolol indicate that a muscarinic receptor is involved.

The ability of simultaneously added carbachol to inhibit part of the stimulation of cyclic AMP by other agonists has been described for several tissues. It can decrease the response to thyrotrophin in thyroid slices (26, 27) and affect several agents that are able to elevate cyclic AMP in 132-1N1 astrocytoma cells (28). Butcher *et al.* (17) have described the immediate effect of carbachol on isoproterenol-stimulated cyclic AMP accumulation in the parotid, which we have confirmed. Butcher[4] has also found that adenylate cyclase isolated from intact parotid cells previously incubated with carbachol cannot be stimulated with catecholamines to the same activity as can normal adenylate cyclase. We have found that the immediate effect in the parotid is not dependent upon extracellular calcium, and thus is not mediated through calcium uptake from the buffer or by cyclic GMP. This is in direct contrast to the finding by Gross and Clark (28) that extracellular calcium was essential for the inhibitory effect in astrocytoma cells, and to the theory of Erneux *et al.* (29) that it is mediated through elevated tissue cyclic GMP in thyroid tissue. Further investigation of the mechanism(s) for the immediate and time-dependent carbachol inhibitory effects needs to be performed.

It has been difficult to state whether the elevation of cyclic AMP is responsible or necessary for the development of cyclic AMP refractoriness. The fact that carbachol can produce only 1% of the maximal cyclic AMP response to isoproterenol does not rule out cyclic AMP as a mediator of refractoriness. However, since a small elevation of cyclic AMP concentration produced by carbachol is a more effective stimulus for the production of a refractory state than is a similar rise in cyclic AMP upon isoproterenol stimulation, at least part of the carbachol effect may be mediated either through unique pools of cyclic AMP not affected by isoproterenol or through mechanisms independent of cyclic AMP. Although isoproterenol is more potent for specific refractoriness than for acute cyclic AMP elevation, it could produce refractoriness through a cyclic AMP-mediated process. This would demand that much of the acute cyclic AMP rise be "overshoot" unnecessary for the induction of refractoriness, as is probably the case for α-amylase release (30). Indeed, the potencies of induction of refractoriness and of α-amylase release relative to cyclic AMP elevation are approximately equal.

There are several possibilities for the step(s) at which cyclic GMP refractoriness is accomplished. The elevation of cyclic GMP by sodium azide does not lead to refractoriness of the carbachol effect. If azide works in the parotid as in other tissues, it acts through a protein factor to stimulate guanylate cyclase directly (31). Furthermore, cyclic GMP refractoriness is unaffected by the removal of extracellular calcium even though the acute elevation of cyclic GMP is completely inhibited.

[4] F. R. Butcher, personal communication.

Thus it seems that refractoriness is not a cyclic GMP-dependent process. Maximal stimulation with either *alpha* adrenergic or muscarinic agonists leads to the same maximal elevation of cyclic GMP. Stimulation with a combination of these two classes of agonist is no more effective than either agent alone. These findings indicate that the elevation of cyclic GMP proceeds through a common mechanism which can be stimulated maximally by either *alpha* adrenergic or muscarinic agonists. Alternatively, separate pools of guanylate cyclase could exist which are linked in such a way that stimulation with either agonist regulates the total cellular cyclic GMP. In either case, since cyclic GMP refractoriness is hormone-specific and seemingly unrelated to the elevation of cyclic GMP, the regulation that leads to this state must occur at a step prior to the convergence of the hormone-induced signals for cyclic GMP synthesis. Such regulation could be accomplished through decreased binding of the agonists at their receptors, which could possibly lead to decreased effect. The refractoriness of potassium release from the parotid to *alpha* adrenergic stimulation (32) has recently been correlated with decreased binding to *alpha* adrenergic receptors (33) in the presence and absence of extracellular calcium. There are other possibilities, perhaps concerning factors as yet undiscovered. Cyclic GMP refractoriness does not seem to be regulated through decreased guanylate cyclase activity, since muscarinic stimulation does not affect the ability of sodium azide to stimulate the accumulation of cyclic GMP.

The rat parotid presents an interesting system for the investigation of cyclic nucleotide refractoriness. Both cyclic AMP and cyclic GMP refractoriness occur, and both involve muscarinic as well as adrenergic receptors. Only one system shows nonspecific refractoriness. The existence of such a cyclic nucleotide pharmacology may be of advantage in the investigation of the mechanisms of refractoriness as well as of the processes mediated by hormones putatively regulated through elevation of parotid cyclic nucleotides.

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