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Amylase Secretion from the Rat Parotid: Refractoriness to Muscarinic and Adrenergic Agonists

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SUMMARY

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The hormone-stimulated secretion of α-amylase (EC 3.2.1.1) from superfused rat parotid tissue has been measured using an automated, continuous system. Stimulation of parotid slices with muscarinic or *alpha* adrenergic agonists produces a rapid initial elevation of the rate of secretion, though the amount of α-amylase secreted is less than with stimulation by the *beta* adrenergic agonist isoproterenol. Isoproterenol-stimulated secretion begins with a slower rate of initial output than does stimulation with either the *alpha* adrenergic agonist phenylephrine or the muscarinic agonist carbachol. If any of these three agonists is allowed to superfuse the tissue continually, α-amylase output reaches a peak rate, then declines over a period of time until it returns to basal rate. Thus continuous stimulation with phenylephrine rapidly produces refractoriness, which is absolute within 20 to 30 min. This refractoriness is specific, since carbachol and isoproterenol, agonists acting through receptors not originally involved, are still active. Continuous stimulation with carbachol or isoproterenol also leads to refractoriness, though the process takes longer than that seen with phenylephrine and needs one to two hours to be completed. Other experiments have shown that the simultaneous stimulation of α-amylase secretion with phenylephrine and carbachol is no more effective than either agonist alone for peak α-amylase secretion. However when α-amylase secretion is stimulated with carbachol plus isoproterenol, more enzyme is released with a greater initial secretion rate than when either agonist is used alone. Further, if isoproterenol stimulation pulses are superimposed upon the continuous superfusion of carbachol, nonspecific refractoriness is seen; carbachol diminishes the ability of isoproterenol to stimulate α-amylase secretion. These forms of secretion refractoriness correlate well with the pharmacology of cyclic nucleotide refractoriness that we have previously reported. However, the ability of cyclic nucleotides to mediate α-amylase secretion is not firmly established. Cyclic AMP may be a second messenger mediating α-amylase secretion. Cyclic GMP, on the other hand, may not play any role in muscarinically- or adrenergically-stimulated α-amylase secretion.

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Indeed, though sodium azide raises the cyclic GMP concentration in parotid slices, it has no effect on α-amylase secretion. This lack of effect is not due to a nonspecific inhibition of secretion. Azide produces little inhibition of carbachol-stimulated α-amylase secretion or cyclic nucleotide accumulation.

INTRODUCTION

Agonists acting through several distinct receptor populations are able to stimulate parotid slices to secrete the enzyme α-amylase (E.C. 3.2.1.1). Isoproterenol interacts with *beta*$_1$ adrenergic receptors to stimulate copious α-amylase secretion (1, 2). Stimulation of *alpha* adrenergic (3) or muscarinic (4) receptors also leads to α-amylase secretion, though with less efficacy than upon isoproterenol stimulation. Several investigators have found that the rate of α-amylase secretion achieved after humoral stimulation cannot be sustained. In each case the rate of secretion eventually slows (5–7). Whether this decline in stimulated secretory rate is due to loss of agonist, depletion of secretable α-amylase, or to a process responsible for the physiological regulation of secretion had never been addressed. We previously detailed the phenomena of cyclic AMP refractoriness and cyclic GMP refractoriness after humoral stimulation of parotid slices (8). The experiments detailed in this report were directed toward answering whether cyclic nucleotide refractoriness is associated with, and perhaps causes the progressive loss of tissue response.

An α-amylase assay which is well suited to the study of secretion time courses has been developed by Matthews *et al.* (9). Using this method, they showed that the initial increase in the secretion rate was greater with *alpha* adrenergic or muscarinic agonists than with *beta* adrenergic stimulation, though greater secretion could ultimately be achieved after isoproterenol stimulation (10, 11). Further, they found that the initial increase in secretion rate was greater with *beta* adrenergic stimulation in the presence of *alpha* adrenergic (10) or muscarinic (11) stimulation than with isoproterenol alone.

We have utilized superfused rat parotid slices to assess secretion dynamics after humoral stimulation. These experiments show that α-amylase secretion cannot be sustained after stimulation with carbachol, phenylephrine, or isoproterenol. Thus, even in the continual presence of superfused secretagogue, α-amylase secretion eventually returns to the basal rate. Agonists that act through receptors distinct from those first stimulated are generally still able to produce some further α-amylase secretion. The tissue has thus become refractory to further stimulation through interaction with an agonist. We have called this phenomenon "secretion refractoriness."[3] In most cases secretion refractoriness correlates well with the analogous cyclic AMP or cyclic GMP refractoriness (8). The possibility that secretion refractoriness is mediated through cyclic nucleotide refractoriness is explored. If cyclic nucleotide refractoriness plays any role in the regulation of physiological processes, it must be shown to lead to refractoriness of a physiological event mediated through increased cellular cyclic nucleotide accumulation.

MATERIALS AND METHODS

Parotid glands were obtained from male Wistar rats (100–200 g) as previously described (6),and preincubated with Krebs-Ringer Bicarbonate buffer containing 6.1 mM D-glucose and 6.1 mM β-hydroxybutyrate (buffer) for 0.5–4 hours at 37°. At various times tissue destined for secretion studies was removed from the incubation vessel and placed into a jacketed superfusion chamber with an internal volume of 1.2 ml maintained at 37°. The tissue was superfused with buffer continuously bubbled with 95% O_2–5% CO_2.

[3] Secretion refractoriness refers to a hormone-induced decrease in the ability of an agonist to stimulate α-amylase secretion. If the tissue is refractory to the agonist originally used to produce α-amylase secretion, specific refractoriness exists. Nonspecific refractoriness exists if stimulation with one agonist produces refractoriness to the ability of another to produce secretion. Cyclic AMP refractoriness and cyclic GMP refractoriness are similarly defined as refractoriness to an agonist's ability to stimulate cyclic nucleotide accumulation.

Drugs were dissolved in glass-distilled water, and added to the main buffer line immediately before being pumped to the superfusion chamber. The drugs were mixed with the oxygenated buffer less than one minute before acting upon the tissue. Water (the drug vehicle) was added to the buffer when no drug was being infused. Throughout these experiments carbachol or phenylephrine was always diluted to a final concentration of 40 μM. A low concentration of propranolol (2.5 μM) was included with phenylephrine to prevent the expression of the latter drug's *beta* adrenergic component on α-amylase secretion (2).

The superfusion effluent was continuously monitored for α-amylase activity by a modification of the method of Matthews *et al.* (9). A fluorogenic substrate, amylopectin anthranilate, was used in the enzyme assay. The substrate solution contained 5 g/liter amylopectin anthranilate in 25 mM sodium phosphate buffer, pH 7.0, along with 25 mM sodium chloride and 0.03% Brij-35. A colloidal suspension of amylopectin anthranilate (20 g/50 ml water) was first dialyzed for two hours against two liters of distilled water. After dialysis the starch was added by drops to the other components which had been heated to 90°. The final solution was then cooled and used at room temperature. The recipient, 25 mM sodium phosphate buffer, pH 7.0, contained 0.03% (v/v) octanoic acid, which was used as the wetting agent in this portion of the flow system in place of Brij-35, since Brij-35 is itself fluorescent. Solutions were pumped through tubes obtained from Acculab, using a 13 channel peristaltic pump from Brinkman/Ismatec (model MP-13).

Once the perfusion effluent was mixed with the starch solution it was pumped through a 50° bath; the transit time was 2.8 min. After this incubation the solution was passed through a 12″ dialyzer fitted with a type C membrane, through which anthranilic acid and its small molecule-linked derivatives pass into the recipient stream. The dialysate then traveled through a flow cell in a Farrand model A-3 fluorometer opened to slit 5 and equipped with 7-60 primary (peak excitatory wavelength at 379 nm) and 3-73 secondary (sharp cut transmission at $\geqq$ 421 nm) filters. The voltage output from a microammeter (1 mV full deflection on the 0.1 μAmp scale) was read on a strip chart recorder (1 mV full scale) which had been zeroed to the tissue-free blank of the starch solution. In each figure, the strip chart numbers refer to percent deflection, with 100% deflection occurring at line 10.

Alpha-amylase assay linearity was assessed by perfusing dilute parotid homogenate through the buffer line of the assay system. The voltage output attained was found to be a linear function of the α-amylase concentration present in up to 7 μg of homogenate protein. This voltage corresponded to a strip chart deflection of 65% of full scale. Only with maximal isoproterenol stimulation did secretion produce peaks of greater than 65% deflection. All other experiments were performed with α-amylase secretion rates that were within the linear range of the assay.

The integrated area under each peak was determined (in μV-sec) using a Hewlett-Packard model 3370B integrator in series between the ammeter and recorder. Ten percent deflection of the strip chart recorder registered 0.16 mV per second. The recorder time constant was provided via the integrator, which was set to noise suppression level 3. Calibration was achieved by infusing a solution of *B. subtilis* α-amylase (0.5 U/ml) through the buffer line in the absence of tissue. Though individual experiments are reported, each result has been obtained at least three times without contradictory evidence. Secretion time courses were quite reproducible (see below), though the magnitudes of response were rather variable even when normalized to the amount of tissue protein.

Parotid tissue on which cyclic nucleotide measurements were to be obtained was prepared and incubated as previously described (8). Tissue preparation for cyclic nucleotide studies was in all ways similar to that described in this manuscript for measurement of secretion, with the exception that all incubations were performed in closed vessels. Incubations were stopped by homogenization of the tissue plus incubation medium in 5% trichloroacetic acid. Supernatants obtained after centrifugation were extracted with diethyl ether, then used in the radioimmunoassay for cyclic

AMP or cyclic GMP (12). The cyclic nucleotides were acetylated by the sequential addition of triethylamine and acetic anhydride to each tissue sample prior to assay, as previously described (13). Samples were measured on the Gamma-Flo automated radioimmunoassay system (14). Cyclic nucleotide acetylation increases both the sensitivity and selectivity of these radioimmunoassays.

Superfused tissue slices were homogenized in 1.0 ml of 5% trichloroacetic acid after completion of each experiment. The pellets obtained after centrifugation for 10 min at full speed in a Sorval GLC-2 benchtop centrifuge were solubilized in 2 ml of 0.5 N NaOH, in preparation for determination of the protein concentration. Protein concentrations of tissue used for cyclic nucleotide determinations were determined on the pellet remaining after centrifugation of the acid homogenate. Protein determinations were performed using the method of Lowry *et al.* (15), adapted to an automated analysis system. Bovine serum albumin was used as the reference protein.

Carbachol chloride, *l*-isoproterenol HCl, *l*-phenylephrine HCl, atropine sulfate, sodium azide and *Bacillus subtilis* α-amylase (1270U/ml) were obtained from Sigma Chemical Company. *l*-Propranolol was a gift of ICI, Ltd., and phentolamine methanesulfonate was provided by Ciba. Amylopectin anthranilate was purchased from Calbiochem. Other compounds were reagent grade or better. None of the drugs used showed any fluorescence under experimental conditions. Rats were obtained from Hilltop Lab Animals, Inc., Scottdale, Pa.

RESULTS

Muscarinic and alpha adrenergic stimulation. A rapid but transient stimulation of α-amylase secretion is achieved by superfusion of parotid tissue with 40 μM phenylephrine (Fig. 1). If superfusion with the drug is continued for longer than 2 min, refractoriness of *alpha* adrenergically-stimulated α-amylase secretion becomes appar-

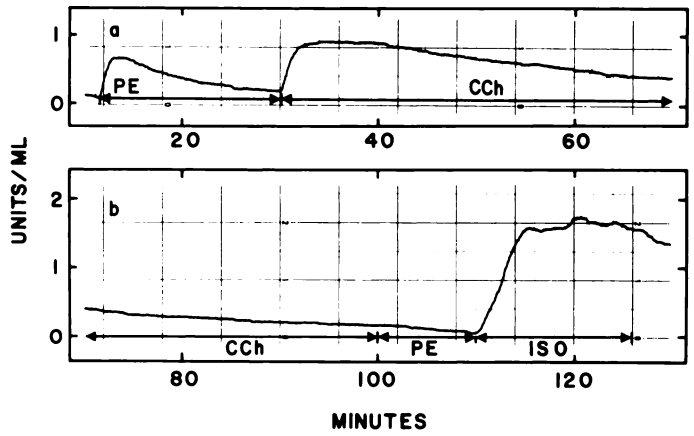

FIG. 1. *Muscarinic and alpha adrenergic refractoriness*

Parotid tissue (0.41 mg protein) was superfused with 40 μM *l*-phenylephrine plus 2.5 μM *l*-propranolol for a period of 18.5 min, at which time the drugs were replaced with 40 μM carbachol. The tissue was superfused with carbachol for 60 min before a fresh solution of the drug was infused for a total carbachol stimulation duration of 70 min. Next, a 10 min superfusion with a fresh solution containing 40 μM *l*-phenylephrine plus 2.5 μM *l*-propranolol demonstrated that the tissue was still refractory to *alpha* adrenergic stimulation. Finally the tissue was superfused with 60 μM *l*-isoproterenol, which was active even though the tissue had just been superfused with 2.5 μM *l*-propranolol. Panel b is the continuation of the record shown in panel a. The abbreviations used are: ISO, *l*-isoproterenol; PE, *l*-phenylephrine; CCh, carbachol. The bars under the record show the length of stimulation with each drug; the drug used is indicated thus in this and all subsequent figures. In each figure the abscissa shows the total superfusion duration (min), while the ordinate is calibrated in units of *B. subtilis* α-amylase per ml of enzyme solution infused through the buffer line.

ent. Within 30 min after the start of continual phenylephrine stimulation, secretion refractoriness is absolute. Refractoriness of α-amylase secretion is not due to destruction of active drug, since application of a fresh phenylephrine solution (near the end of this experiment) was ineffective. It is specific for *alpha* adrenergic agonists, since both carbachol (40 μM) and isoproterenol (60 μM) are able to produce enzyme secretion from tissue refractory to phenylephrine. This also proves that α-amylase secretion refractoriness after phenylephrine cannot be accounted for by depletion of the cell's secretable α-amylase, which by itself might have been the reason for a time-dependent decline in α-amylase secretion.

As can also be seen in the experiment shown in Fig. 1, carbachol stimulated α-amylase secretion becomes refractory to further carbachol stimulation after a sufficient period of tissue superfusion. Interestingly, carbachol must stimulate the tissue for over one hour to produce full refractoriness, an interval more than twice as long as needed with *alpha* adrenergic stimulation. This is seen whether or not the tissue had been stimulated with phenylephrine prior to the addition of carbachol. Thus continual *alpha* adrenergic or muscarinic stimulation cannot produce sustained α-amylase secretion. Secretion stimulated by phenylephrine quickly reaches its maximal rate, then declines. The rate of this decline ("decline time") was quantified by measuring the time from the initiation of humoral stimulation until the secretion rate had returned from its peak to half of the peak stimulation above basal. The decline time was found to be 10 ± 2 min after phenylephrine stimulation (n = 3, mean ± standard error), and 30 ± 5 minutes upon stimulation with carbachol (n = 4). Thus refractoriness occurs more quickly after *alpha* adrenergic than muscarinic stimulation. Refractoriness following carbachol stimulation is not due to depletion of α-amylase, since isoproterenol is able to stimulate some further secretion under these conditions (Fig. 1; see also Fig. 8). The stimulation with isoproterenol seen at the end of the experiment shown in Fig. 1 is not as great as would be expected of fresh tissue. It may be reduced in Fig. 1 because of the prior exposure to 2.5 μM propranolol, to α-amylase depletion, or to nonspecific refractoriness (see below).

We previously showed that cyclic GMP accumulation is no greater after stimulation of parotid tissue with the combination of carbachol and norepinephrine than with either agonist alone (8). This is also the case with α-amylase secretion. Figure 1 shows that approximately equal peak secretion rates are seen upon stimulation with either carbachol or phenylephrine. We have also confirmed the observation that phenylephrine and carbachol stimulate *alpha* adrenergic and muscarinic receptors, respectively (10, 11). However, these agonists used together are no more effective for initial stimulation of α-amylase secretion than is carbachol alone (Fig. 2). The agonists produce neither additive secretion nor additive cyclic GMP accumulation.

Role of Cyclic GMP in Secretion. Though correlations of α-amylase secretion with cyclic GMP can be observed, the ability of cyclic GMP to mediate secretion has not been proven. Indeed, our evidence suggests that cyclic GMP has no such role. Sodium azide (300 μM) has previously been found to elevate cyclic GMP in our hands (8). However, superfusion of tissue with this drug does not alter the basal α-amylase secretion rate. Further, azide has only a small effect on the normal response to carbachol (Fig. 3).Though the peak effect of a one minute pulse with 40 μM carbachol is smaller during the final minute of an 11 min treatment with 300 μM sodium azide than before, it is 75% of normal. This proves that secretion can still be stimulated by secretagogues, though sodium azide (and thus elevated cyclic GMP) could not stimulate secretion from this tissue. The same observation has also been made using isoproterenol or phenylephrine for the final minute of an 11 min stimulation with sodium azide (data not shown). Table 1 shows that concurrent sodium azide does not affect the ability of various agonists to elevate cyclic GMP. In fact, the cyclic GMP elevation appears additive. Azide may slightly affect the ability of isoproterenol to raise cyclic AMP.

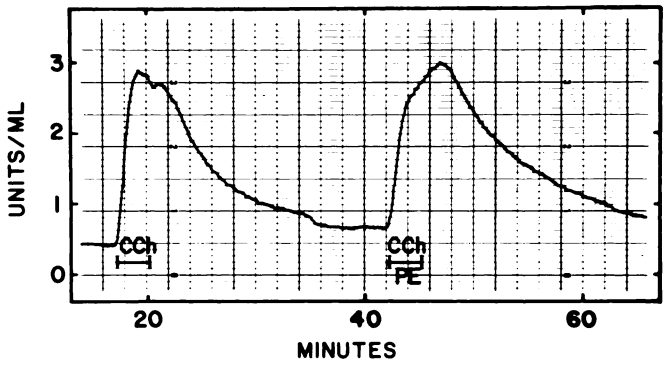

FIG. 2. *Combined carbachol-phenylephrine stimulation*

Parotid tissue (0.34 mg protein) was stimulated with a 3 min pulse of carbachol (40 μM), followed by a 22-min rinse. At that time the tissue was stimulated with a 3 min pulse of carbachol and phenylephrine (each 40 μM), along with 2.5 μM propranolol. The abbreviations used are: CCh, carbachol; PE, phenylephrine.

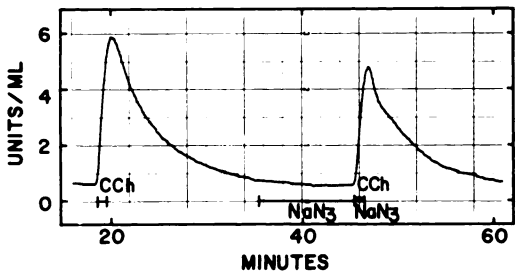

FIG. 3. *Lack of ability of azide to stimulate α-amylase secretion*

Parotid tissue (0.84 mg protein) was stimulated with a 1 min pulse of 40 μM carbachol, which was subsequently washed out with buffer, then superfused for 11 min with 300 μM sodium azide. During the final minute of this period a second 1 min carbachol pulse (40 μM) was performed. Abbreviations: CCh, carbachol chloride; NaN_3, sodium azide.

Beta adrenergic stimulation. Secretion refractoriness also develops upon stimulation with *beta* adrenergic agonists as shown in Fig. 4. Isoproterenol (1.2 μM) stimulates a large increase in secretion rate, but the stimulation is transient. With time, the secretion rate declines even in the continued presence of isoproterenol, until it eventually returns to the basal level. The lack of response to isoproterenol is not due to loss of drug through metabolism or oxidation, since a fresh solution of isoproterenol is equally ineffective. Other experiments with tissue refractory to 1.2 μM isoproterenol have shown that even 12 μM isoproterenol is ineffective after refractoriness has developed. Secretion produced by stimulation with maximal isoproterenol is great enough that simple depletion of α-amylase could account for the reduced secretion. Total depletion of secretable α-amylase does not occur, since phenylephrine is still somewhat able to stimulate secretion after absolute refractoriness to isoproterenol has developed. The finding that any secreton can be obtained by an agonist acting through a receptor population other than the *beta* adrenergic one stimulated by isoproterenol is evidence that true refractoriness rather than α-amylase depletion is present. Further, we found that only 70% of the tissue's α-amylase content is secreted before nearly complete refractoriness to isoproterenol had developed. Measurements of cellular α-amylase remaining in tissue homogenates after isoproterenol stimulation may reflect the amount of enzyme available for secretion. If so, depletion of α-amylase cannot be instrumental for the development of secretion refractoriness. Depletion of the enzyme probably does contribute to the observed decline in secretion rate, however. Therefore, further experiments directed toward eliminating depletion as a contributing factor for secretion refactoriness were peformed.

If secretion refractoriness occurs upon continual isoproterenol stimulation, such refractoriness should be apparent even with isoproterenol concentrations low enough to prevent interference from depletion of α-amylase. Thus, as shown in Fig. 5, 0.02 μM isoproterenol eventually produces refrac-

TABLE 1

Effect of azide on cyclic nucleotide accumulation

The accumulation of cyclic AMP and cyclic GMP (as -fold of basal, mean ± standard error of three experiments, each performed with triplicate samples) after various treatments is shown. The first teatment was given for 12 min, and the second for the final 2 min of that incubation; if no treatment is listed, nothing was added at the time studied. The mean basal cyclic AMP concentration was 4.48 ± 0.86 pmol/mg protein, while for cyclic GMP it was 134 ± 33 fmol/mg protein. The abbreviations used are: ISO, *l*-isoproterenol HCl; CCh, carbachol chloride; NaN_3, sodium azide.

First Treatment	Second Treatment	Cyclic AMP	Cyclic GMP
12 min	*2 min*	*-fold/basal*	*-fold/basal*
—	50 μM ISO	15.8 ± 1.1	1.05 ± 0.09
—	40 μM CCh	1.22 ± 0.09	1.38 ± 0.19
—	40 μM CCh + 50 μM ISO	7.34 ± 1.0	1.53 ± 0.08
300 μM NaN_3	—	0.99 ± 0.10	1.81 ± 0.23
300 μM NaN_3	50 μM ISO	12.1 ± 1.7	2.19 ± 0.26
300 μM NaN_3	40 μM CCh	1.49 ± 0.33	2.66 ± 0.12

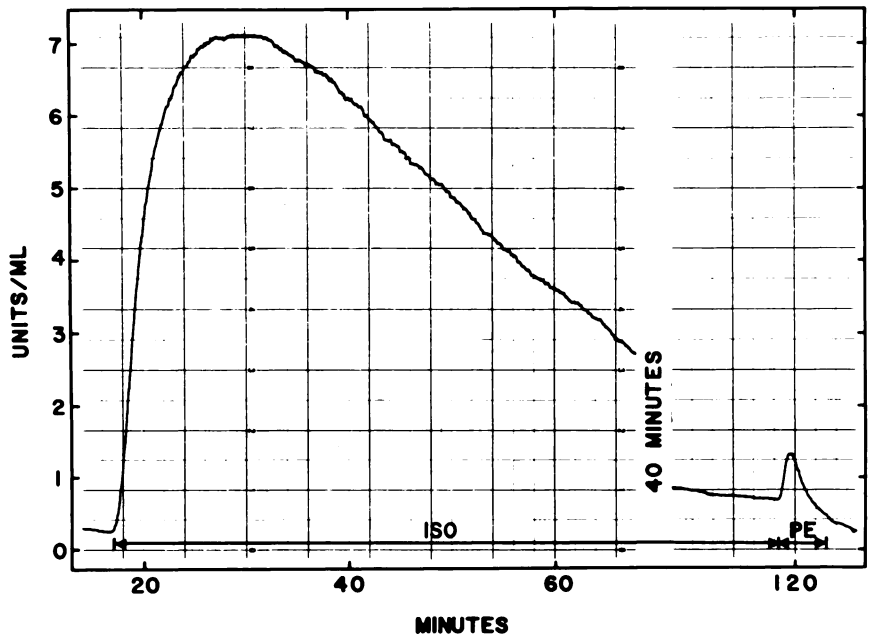

FIG. 4. *Beta adrenergic refractoriness (maximal dose)*

Parotid tissue (0.64 mg protein) was superfused with 1.2 μM *l*-isoproterenol for 85 min, at which time a fresh solution of 1.2 μM isoproterenol was substituted. That fresh solution superfused the tissue for 25 min more before it was exchanged for 40 μM *l*-phenylephrine plus 2.5 μM *l*-propranolol, which was used for 4 min before a no drug rinse was begun. The middle 40 min of α-amylase output during the isoproterenol stimulation are not shown, but consisted of a gradual persistent decline to the activity seen after the break in the figure. Abbreviations are as in Fig. 1.

toriness to further stimulation with that same concentration. The length of time necessary to produce refractoriness to low doses of isoproterenol is longer than with *alpha* adrenergic or muscarinic stimulation; the return to half-maximal secretion rate (decline time) requires 92 ± 10.7 min upon continual stimulation with 0.01 μM isoproterenol (n = 3). Figure 5 further shows that higher concentrations of isoproterenol are still able to produce α-amylase secretion, which proves that total depletion of α-amylase is not responsible for the observed refractoriness. Some reduction of

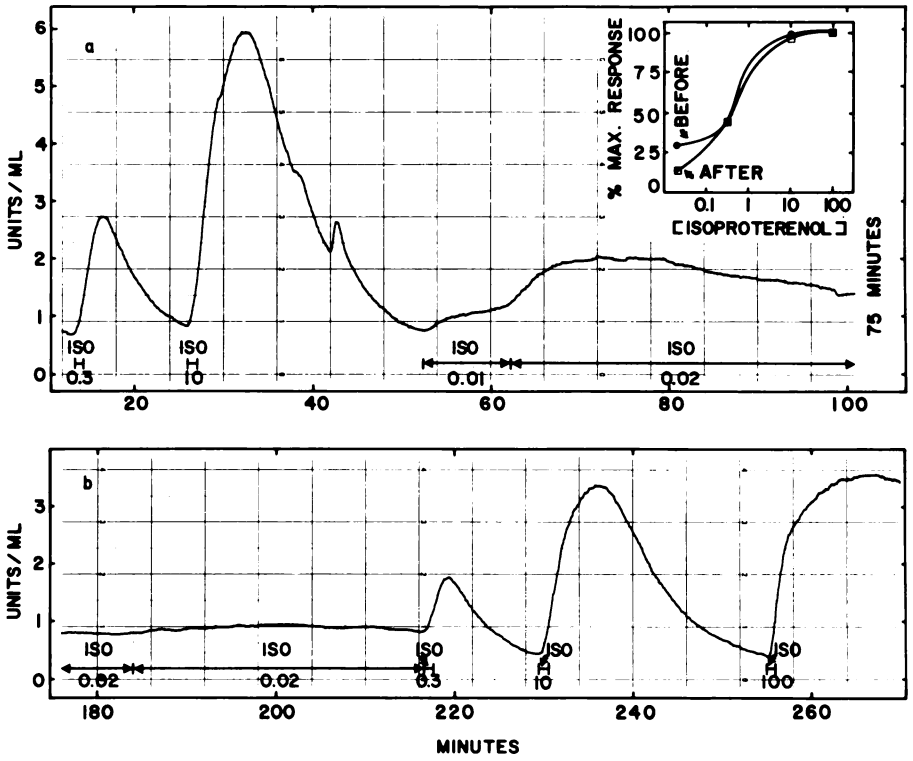

FIG. 5. *Beta adrenergic refractoriness (low dose)*

Parotid tissue (0.67 mg protein) was stimulated with 1 min pulses of isoproterenol; the concentrations used (in μM) are indicated below the stimulation indicator bars. It was then superfused with 0.01 μM *l*-isoproterenol for 10 min, then 0.02 μM for 125 min, at which time a fresh isoproterenol solution (0.02 μM) was substituted. The fresh solution superfused the tissue for 33 min (with minimal effect), at which time the tissue was again stimulated with 1 min isoproterenol pulses. The middle 75 min of the first 0.02 μM isoproterenol stimulation are not shown, but consisted only of a gradual decline of α-amylase output to the level shown after the break in the figure. Panel b is the continuation of the record shown in panel a. Abbreviations are as in Fig. 2.

maximal response to isoproterenol does develop during the two hours necessary to produce refractoriness, as shown by the responses to one minute pulses of various isoproterenol doses both prior to and after refractoriness (Fig. 5).

If the responses are normalized to percentage of maximal response, refractoriness to a low dose of isoproterenol does not alter isoproterenol's potency for secretion. Nevertheless, even with normalization it is apparent that refractoriness to at least the low isoproterenol dose originally used exists (inset, Fig. 5). Thus *beta* adrenergic refractoriness exists even with secretion far below the rate needed to deplete α-amylase, though two controls are necessary to prove that this shift in isoproterenol response is not simply due to prior secretion. An isoproterenol dose-response relationship, determined both before and after a superfusion period as long as that used to produce refractoriness in Fig. 5, shows that neither the length of incubation nor the fact of prior secretion produces refractoriness *per se* (Fig. 6). Thus tissue was treated with pulses of isoproterenol in increasing doses, similar to the procedure used to show refractoriness to the low isoproterenol dose, then superfused for nearly two hours in the absence of any drug. At the end of this treatment the tissue still possessed full sensitivity to isoproterenol, unlike tissue made refractory upon superfusion with 0.02 μM isoproterenol. The decline in efficacy to 60% normal, which may represent depletion of secretable α-amylase, is reflected in all doses of isoproterenol tested (see inset in

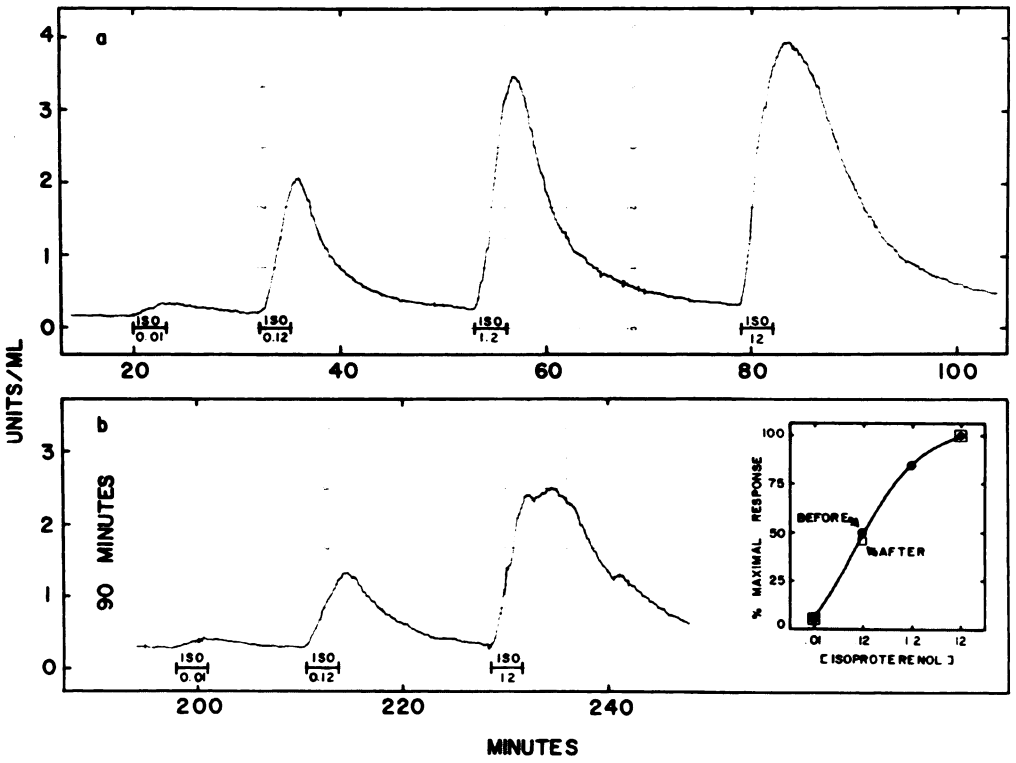

FIG. 6. *Isoproterenol-stimulated α-amylase release*

An isoproterenol dose-secretion study on parotid tissue (0.46 mg protein) was performed using successive 3 min pulses with *l*-isoproterenol at the following concentrations (in μM): 0.01, 0.12, 1.2, 12. The drug infusions were interspersed with no drug rinses. After these 4 stimulations, the tissue was superfused for 115 min in the absence of any agonist, the final 90 min of which are not shown. During that period the α-amylase output slowly declined to the level shown in panel b. After this rinse, a second dose-secretion study was performed on this tissue, again using 3 min pulses with the drug (0.01, 0.12, 12 μM). Panel b is a continuation of the experiment shown in panel a, though it begins 90 min after the end of the record shown in panel a. Drug concentrations are denoted (in μM) underneath the stimulation indicator bars. The inset shows this log dose-response relationship both before and after the 2 hr rinse period; the ordinate is plotted as percentage of maximal response in each set. The abbreviation used is: ISO, *l*-isoproterenol.

Fig. 6) and is similar in magnitude to the decline seen with low isoproterenol dose secretion refractoriness as shown in Fig. 5. The initial dose-response experiment shown in Fig. 6 caused secretion of α-amylase activity which generated a total integrated area of 19.3 V-sec/mg protein, compared to 21.8 V-sec/mg protein generated by the continuous superfusion with 0.02 μM isoproterenol and initial secretion with pulses of isoproterenol shown in Fig. 5. Thus a similar amount of α-amylase depletion would have occurred in each. Neither depletion nor perfusion time account for the observed refractoriness after perfusion with doses of isoproterenol. That repetitive pulses of isoproterenol (1.2 μM) do not by themselves produce refractoriness is shown in Fig. 7. At least four cycles of stimulation are possible before any significant loss of peak effect or total secretion (integrated area) is noticeable.

Influence of carbachol on isoproterenol-stimulated secretion. Carbachol stimulation rapidly leads to a maximal rate of α-amylase secretion, while isoproterenol more slowly produces secretion which ultimately becomes greater than that achieved with carbachol.

α-Amylase secretion stimulated by isoproterenol is affected by the copresence of carbachol. Carbachol (40 μM) plus isopro-

terenol (1.2 μM) produces more secretion than could either drug alone (Fig. 8). Further, the peak of secretion is reached more quickly when the drugs are combined than with isoproterenol alone, reflecting carbachol's ability to stimulate a greater initial rate of α-amylase secretion than can isoproterenol. We have also confirmed the observation (10) that simultaneous *alpha* and *beta* adrenergic stimulation leads to a similar large but rapidly increased secretion rate (not shown).

Once carbachol has stimulated the tissue for 20 min or more, isoproterenol's ability to stimulate α-amylase secretion becomes partially refractory; comparing Fig. 8 with the previous figure shows that less than expected secretion occurs. When continual carbachol stimulation is superimposed upon pulsed isoproterenol additions, refractoriness of both carbachol's sustained α-amylase secretion and isoproterenol's stimulation is seen. The later isoproterenol-mediated release peaks do not exhibit the rapid initial secretion rate which presumably was due to carbachol when it first interacted with isoproterenol. While depletion of secretable α-amylase may account

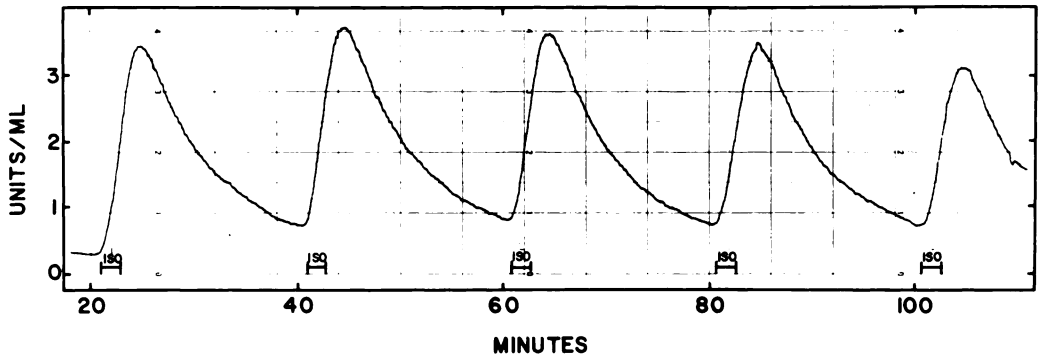

FIG. 7. *Repetitive isoproterenol stimulation*

Five cycles of isoproterenol (1.2 μM) for 2 min followed by 18 min washout periods were used to stimulate α-amylase secretion from parotid tissue (0.78 mg protein). The integrated areas for the five peaks are (in V-sec) 2.8, 3.0, 2.8, 2.7 and 2.2, respectively. The abbreviation used is: ISO, *l*-isoproterenol.

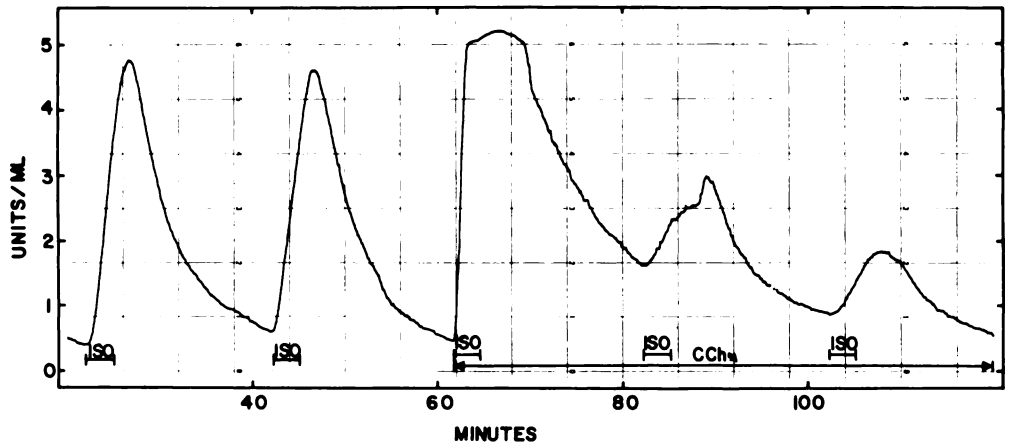

FIG. 8. *Effect of carbachol on isoproterenol-stimulated secretion*

Parotid tissue (0.41 mg protein) was superfused for two cycles of 1.2 μM *l*-isoproterenol for 3 min followed by 16 min without stimulation. At the end of the second cycle the tissue was stimulated with the combination of 1.2 μM *l*-isoproterenol and 40 μM carbachol for 3 min, followed by stimulation with carbachol for the remainder of the experiment. Only at specific times was isoproterenol added along with the carbachol, and when added was used for 3 min pulses at 1.2 μM. The abbreviations used are: CCh, carbachol; ISO, isoproterenol.

for some of the observed decline, it cannot explain all of it. The time-dependent effect of carbachol on isoproterenol-stimulated α-amylase secretion was also observed in the absence of a large prior carbachol plus isoproterenol-stimulation secretion (Fig. 9). Whereas the repetitive isoproterenol pulses in Fig. 7 generated a total integrated area of 17.3 V-seconds/mg protein, the secretion shown in Fig. 9 generated only 13.4 V-seconds/mg protein up to the point of the second isoproterenol stimulation. Enough secretion to produce 24.7 V-seconds/mg protein had been secreted by the time of the third isoproterenol application. At that time isoproterenol could stimulate little further secretion. Dibutyryl cyclic AMP was still able to produce appreciable α-amylase secretion at that time (Fig. 9). Therefore, it appears that the time-dependent effect of carbachol on isoproterenol-mediated α-amylase secretion does not depend entirely upon depletion of secretable α-amylase.

DISCUSSION

Alpha-Amylase secretion from rat parotid tissue can be stimulated through *alpha* adrenergic, *beta*$_1$-adrenergic, and muscarinic receptors (1–4). As we have shown, the rate of α-amylase secretion slows with

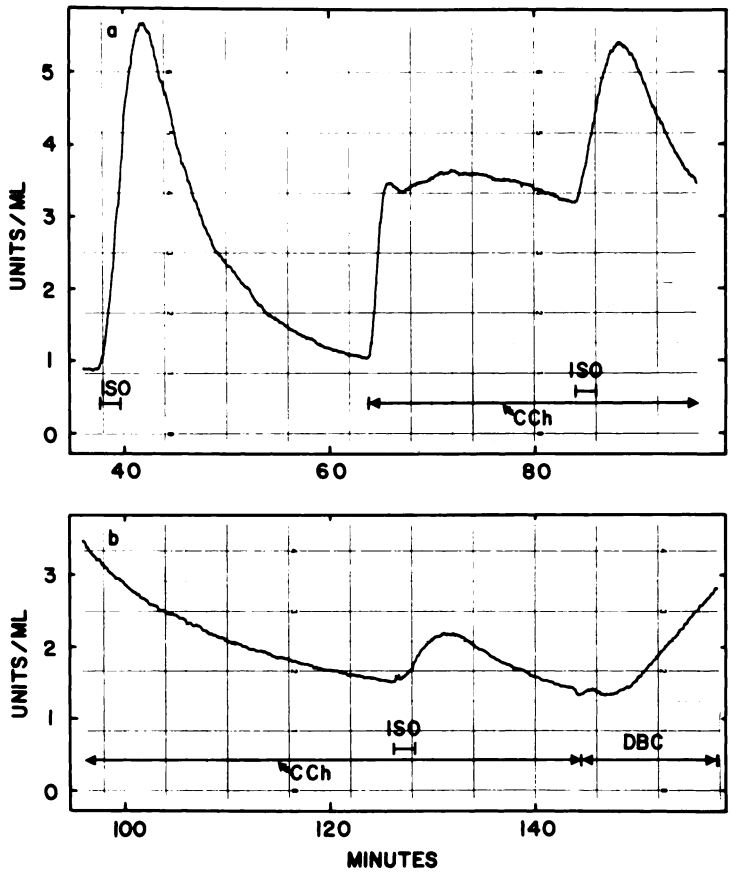

FIG. 9. *Time-dependent effect of carbachol on isoproterenol-stimulated α-amylase secretion*

Parotid tissue (0.80 mg protein) was stimulated first with a 2 min isoproterenol (10 μM) pulse, followed by a 24 min rinse in the absence of any agonist. At the end of this time an infusion of 40 μM carbachol was begun. After 20 and 60 min of this infusion further 2 min isoproterenol pulses were performed concurrent with the carbachol infusion. Carbachol was continued for a total of 78 min, after which infusion with 2 mM dibutyryl cyclic AMP was begun. The abbreviations used are: ISO, *l*-isoproterenol; CCh, carbachol; DBC, dibutyryl cyclic AMP. This one experiment was not repeated.

time. Whether the secretory rate decrease is due to loss of agonist, depletion of α-amylase, or secretion refractoriness had never been addressed. We have previously detailed the phenomena of cyclic AMP refractoriness and cyclic GMP refractoriness in rat parotid. The experiments detailed in this report were directed toward answering whether cyclic nucleotide refractoriness is associated with and perhaps causes α-amylase secretion refractoriness.

Cyclic AMP is thought to mediate isoproterenol's ability to stimulate α-amylase secretion. *Beta* adrenergic agonists stimulate cyclic AMP accumulation (16). Further, isoproterenol-stimulated secretion is potentiated by theophylline and mimicked by dibutyryl cyclic AMP (17). The results of this research indicate that there is a good correlation between cyclic AMP refractoriness and secretion refractoriness after isoproterenol stimulation. A cause-effect relationship between the two may exist, though it has not been proven.

Cyclic AMP refractoriness begins to develop soon after stimulation of parotid slices with a maximal isoproterenol concentration (8). The process is not complete even after three hours, though by that time the cyclic AMP accumulation is less than one-fourth of that at the peak. Cyclic AMP refractoriness development has also been found to be dose-dependent. Low doses of isoproterenol produce only partial or no refractoriness to subsequent maximal-dose *beta*-adrenergic stimulation, while full refractoriness is produced only with a primary dose of approximately 1 μM *l*-isoproterenol (8). *Alpha*-Amylase secretion refractoriness correlates well with most of these observations. High doses of isoproterenol produce total α-amylase secretion refractoriness to itself, which does not fully restrict phenylephrine's ability to produce secretion. Low doses of isoproterenol produce full refractoriness to that same dose, though higher doses are still somewhat effective. It is hard to determine the true time course of secretion refractoriness, especially with higher isoproterenol doses, since α-amylase depletion undoubtedly contributes to the observed decline in secretory rate. Nevertheless, the length of time necessary is consistent with the time course of cyclic AMP refractoriness, at least with submaximal isoproterenol concentrations.

Nonspecific cyclic AMP refractoriness also exists. Thus carbachol can reduce isoproterenol's ability to elevate cyclic AMP. Carbachol's effect on isoproterenol-stimulated cyclic AMP accumulation is composed of two distinct effects: acute and time-dependent (8). Carbachol (20 μM) produces an average 36% inhibition of acute cyclic AMP accumulation as well as a further 50% reduction in the remainder with a 30 min preincubation.

One relationship between cyclic nucleotide metabolism and secretion refractoriness does not correlate. The initial effect of carbachol and isoproterenol on α-amylase secretion is qualitatively additive, though it is inhibitory for cyclic AMP accumulation. Thus the rate of α-amylase secretion in the presence of both agonists is greater than would be expected of either one alone (Fig. 8). This observation has previously been reported by Petersen and Ueda (10, 11), using superfused mouse parotid segments. They suggested that carbachol's effect was due to its ability to stimulate both fluid and α-amylase secretion, while isoproterenol produces only a viscous protein solution. Increased fluid secretion from the acinar cells would be expected to help clear secreted α-amylase through the parotid ducts. It is also possible that the combination of agonists actually causes greater α-amylase secretion from acinar cells. If so, the mechanism may be related to the observations of Sharoni *et al.* (18). They found that when parotid tissue is stimulated by isoproterenol "old" α-amylase is preferentially secreted before "newly synthesized" (pulse-labeled) protein. Muscarinic or *alpha* adrenergic agonists added together with isoproterenol greatly increase randomization of secretion, so that the cell makes no distinction between "new" and "old" enzyme. This would make more enzyme immediately available for secretion, and may increase the initial secretory rate.

The fact that the initial stimulation with carbachol plus isoproterenol leads to increased secretion but decreased cyclic AMP accumulation does not necessarily disso-

ciate cyclic AMP from α-amylase secretion. It is possible that the acute decrease in cyclic AMP concentration is "nonfunctional," being in excess of that necessary for maximal effect, or a nonfunctional pool of cyclic AMP that is affected by the acute addition of carbachol. Indeed, recent studies by Terasaki and Brooker (19) have shown that in rat cardiac tissue cyclic AMP accumulation stimulated by isoproterenol to only 4-fold of basal levels produces essentially maximal binding to intracellular cyclic AMP binding sites. While they were able to elevate cyclic AMP more than 20-fold by including a phosphodiesterase inhibitor, bound cyclic AMP only remained at 4-fold of basal. This suggests that 26-fold elevations seen in parotid tissue are indeed supramaximal, as does the fact that isoproterenol is more potent for α-amylase secretion than for cyclic AMP accumulation (2).

Carbachol also produces a time-dependent nonspecific cyclic AMP refractoriness which inhibits isoproterenol stimulated cyclic AMP accumulation after a 1 to 60 min preincubation with parotid tissue (8). Similar time-dependent nonspecific refractoriness is apparent in α-amylase secretion as well (Fig. 8). This effect correlates with cyclic AMP refractoriness, but may be due to other mechanisms. It does not seem to be due to α-amylase depletion, since an experiment similar to that shown in Fig. 8, but without the large initial α-amylase secretion, produced similar nonspecific secretion refractoriness (Fig. 9). It may be related to carbachol's stimulation of fluid or potassium secretion, or to its ability to produce vacuoles within the acinar cells (20).

The link between cyclic GMP accumulation and α-amylase secretion is quite tenuous. *Alpha* adrenergic and muscarinic agonists stimulate both α-amylase secretion (3, 4) and cyclic GMP accumulation (8, 21). However, we have found that sodium azide, which also stimulates cyclic GMP accumulation (8), does not produce α-amylase secretion (Fig. 3). Thus it is unlikely that cyclic GMP accumulations mediate α-amylase secretion unless azide stimulates a pool of cyclic GMP which is different from that affected by carbachol or norepinephrine. A direct role of cyclic GMP on α-amylase secretion is not proved, and it is probable that the two processes are independently regulated through a more proximal intermediate. Nevertheless, as the data presented in this report show, there is a good relationship between cyclic GMP refractoriness (8) and α-amylase secretion refractoriness. Neither carbachol nor phenylephrine are able to sustain the elevation of cyclic GMP (8). This represents specific refractoriness, since after initial stimulation with phenylephrine, carbachol is still fully active when phenylephrine is unable to alter the cyclic GMP concentration. Likewise, carbachol-produced cyclic GMP refractoriness applies only to carbachol. Figure 1 shows that such a pattern of specific refractoriness exists for α-amylase secretion, though secretion refractoriness seems to become absolute more rapidly than does cyclic GMP refractoriness after either *alpha* adrenergic or cholinergic stimulation.

The inability of phenylephrine or carbachol to sustain α-amylase secretion could be explained by a mechanism other than actual refractoriness. If each agonist stimulated separate pools of α-amylase that were rapidly depleted it would appear as specific refractoriness. Each agonist would produce refractoriness against further stimulation by itself but not other agonists. However, such a mechanism would also predict that stimulation with the combination of carbachol and phenylephrine would lead to greater α-amylase secretion than with either agonist alone. This was not observed (Fig. 2). Further, at least 90% of the tissue's α-amylase activity can be secreted by isoproterenol under optimal conditions (22). The rate of carbachol-stimulated secretion is about one-third, and the rate of phenylephrine-stimulated secretion about one-fifth as rapid as that produced by isoproterenol (23). These fractions imply that separate pools cannot exist. No evidence supporting independent pools exists.

Of course correlations do not prove cause-effect relationships. The possible roles of either cyclic AMP or cyclic GMP to produce α-amylase release are not firmly established. Indeed, release and cyclic nucleotide accumulation refractoriness may only be independent markers of the same

prior event. However; nothing found in our studies suggests that a physiological role for cyclic AMP refractoriness in α-amylase release does not exist, at least in the range of cyclic AMP concentrations that are not supramaximal.

Similar correlations have been found in a few other tissues. Plas and Nunez (24) investigated cyclic AMP refractoriness and its attendant refractoriness of glycogenesis in cultured fetal liver cells. Cyclic AMP accumulation was transient with continual glucagon stimulation in this tissue, peaking at 20-fold of basal within 2 min. After four hours' exposure to the drug, cyclic AMP had fallen to 25% of the peak value, and glucagon was no longer able to elevate the cyclic nucleotide. Similarly, the glucagon-stimulated decrease of [^{14}C]glucose incorporation into glycogen became refractory to glucagon, but remained normally responsive to norepinephrine. Specific refractoriness of decreased glucose incorporation existed at the same time as cyclic AMP refractoriness to glucagon though the ability of norepinephrine to elevate cyclic AMP was not tested. Higgins (25) found that isolated clam ventricles stimulated with a supramaximal dose of 5-HT over a 10 hour period became refractory to the drug's ability to increase heart contractility and cyclic AMP concentration. The decline of inotropism paralleled cyclic AMP refractoriness with respect to both time of development and degree of refractoriness specificity between 5-HT and a neuropeptide.

While such correlations do not prove that cyclic nucleotides regulate various of the physiological responses stimulated by hormones, they are necessary to show that cyclic nucleotide refractoriness plays a role in the regulation of tissue response to humoral agents. Further, elevation of cyclic nucleotide concentration is probably an early event, stimulated by the same agonists which ultimately produce the physiological end effect. Irrespective of the direct role of cyclic nucleotides to produce that end effect, refractoriness of their elevation probably relates to all processes stimulated as an early event after hormone-receptor interaction. Thus cyclic nucleotide refractoriness measures events that are directly or indirectly related to the physiological end effect. We have now established in the rat parotid an experimental system well suited to correlations between cyclic nucleotide and end response refractorinesses, and indeed the pharmacology of the two refractoriness processes are well correlated. While cyclic GMP may not directly mediate α-amylase secretion, it probably reflects some process which does. Cyclic AMP elevation probably does mediate α-amylase secretion. In both cases we have now established that the early manifestation of cyclic nucleotide refractoriness is related to secretion refractoriness, and thus could be one of several processes responsible for the regulation of this physiological event.

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