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INTERACTION BETWEEN THE CALCIUM AND CYCLIC AMP MESSENGER SYSTEMS IN PERIFUSED RAT PAROTID ACINAR CELLS

POSSIBLE MECHANISM FOR POTENTIATION OF AMYLASE SECRETION

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Abstract—Potentiation of amylase secretion induced by a combination of isoproterenol and carbamylcholine was examined in perifused rat parotid acinar cells. The time course of changes in the augmented amylase secretion induced by isoproterenol plus carbamylcholine was similar to that induced by carbamylcholine alone, but not to that caused by isoproterenol. Concentration–response analysis showed that isoproterenol increased the apparent affinity for carbamylcholine to stimulate amylase secretion with the maximum effect attained by isoproterenol plus carbamylcholine being higher than that attained by isoproterenol or carbamylcholine. 8-Bromo cyclic AMP, forskolin and 3-isobutyl-1-methylxanthine mimicked the effect of isoproterenol. Calcium ionophores (A23187 and ionomycin), but not phorbol 12,13-dibutyrate, mimicked the effect of carbamylcholine. Chelation of intracellular free calcium with 1,2-bis-[2-aminophenoxyl]-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, but not that of extracellular calcium with [ethylenebis(oxyethylenenitrile)]-tetraacetic acid (EGTA), abolished the potentiation. Calmodulin antagonists inhibited amylase secretion induced by isoproterenol plus carbamylcholine or carbamylcholine alone, but not that induced by isoproterenol alone. These results suggest that the potentiation is mainly, if not completely, caused by a coordinated interaction between the cyclic AMP system and the Ca^{2+} system at a step distal to second messenger generation, probably via a cyclic AMP-induced increase in the sensitivity of the Ca^{2+} response element to calcium.

There appear to be at least two basic mechanisms that mediate agonist-induced amylase secretion in the rat parotid gland. Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) represents a major messenger system that links hormone–receptor activation to α-amylase secretion [1, 2]. The β-adrenergic agonist isoproterenol causes a large increase in amylase secretion by stimulating the generation of cellular cyclic AMP [3, 4]. Muscarinic cholinergic and α-adrenergic agonists cause the elevation of a cytosolic free calcium concentration ($[Ca^{2+}]_i$), which is due to inositol-1,4,5-trisphosphate (IP_3) mediated release of calcium from intracellular stores as well as the influx of calcium from extracellular fluid [1, 5, 6]. The large rise in $[Ca^{2+}]_i$ induced by carbamylcholine is usually accompanied by only a very small increase in amylase secretion [1, 7]. This view, however, is mostly based on results obtained in batch systems, in which the effects of these agonists on amylase secretion were examined by incubating the cells or slices in flasks for various times. In perifused parotid cells, we have already reported [8] very rapid and marked development of refractoriness of amylase secretion to carbamylcholine, but not to isoproterenol. This result shows that batch systems are not ideal for analysis of the mechanism of amylase secretion induced by carbamylcholine.

There are several reports that examine the potentiation of amylase secretion induced by combined additions of carbamylcholine and isoproterenol [9–11], but the intracellular mechanism underlying the potentiation is not yet known. McKinney and Rubin [11] reported the enhancement of amylase secretion induced by a combination of cyclic AMP and phorbol 12,13-dibutyrate (PDBu†), and suggested the involvement of protein kinase C in this potentiation.

In the present experiment we used perifused parotid acinar cells to study the potentiation of amylase secretion induced by combined additions of isoproterenol and carbamylcholine. Our results suggest that the potentiation of amylase secretion by a combination of isoproterenol and carbamylcholine cannot be explained by synergistic interaction between cyclic AMP and protein kinase C and that it is mainly, if not completely, caused by the cyclic AMP system-mediated increase in the sensitivity of the calcium response to calcium.

METHODS

Preparation of parotid cells. Male Wistar (Gunma)

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† Abbreviations: PDBu, phorbol 12,13-dibutyrate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrile)]-tetraacetic acid; and BAPTA-AM, 1,2-bis [2-aminophenoxy] ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

rats (300–430 g), which had been given free access to food and water, were killed by cervical dislocation. The parotid glands were trimmed of fat, connective tissues and blood vessels and cut into small pieces with scissors. Unless otherwise stated, the preparation and perifusion medium consisted of modified Krebs Ringer phosphate buffer (127.5 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM sodium phosphate), pH 7.4, containing 10 mM glucose, 5 mM β-hydroxybutyrate, 5 mM glutamate, and 0.1% bovine serum albumin. The pieces were transferred into 5 mL of the medium without $CaCl_2$ and $MgSO_4$ containing 3.5 mg collagenase, 1 mg hyaluronidase, and 1% bovine serum albumin, and incubated at 37° for 60 min with shaking in a 100-mL PMP Erlenmyer flask. After incubation, the pieces were dispersed by sucking up and down in a 5-mL polyethylene pipette. The dispersed suspensions of acini were passed through a 148-μm nylon mesh and centrifuged at 50 *g* for 5 min. The acini were washed twice by altered suspension and centrifugation and finally suspended in 5 mL of the medium containing 1% bovine serum albumin. Just prior to each experiment, 0.5-mL aliquots of the cell suspensions [about 1.0 mg (1.03 ± 0.03 mg; N = 26) cell protein] were washed and resuspended in fresh medium and used for each perifusion. The cell preparations were about 90% viable, as determined by trypan blue exclusion.

Perifusion. The perifusion chamber used in this experiment consists of 1-mL micropipette tips sealed with cotton wool in the tip containing an 80-μL bed of Bio-Gel P-2 (200–400 mesh) resin (Bio-Rad Laboratories). The parotid cells, mixed with a 200-μL bed of the gel, were layered on the resin. The top of each cell layer was covered with about 200 μL of medium during the perifusion to prevent air from entering into the supporting matrix. Bio-Gel P-2 resin, used as a matrix for the cells, was swollen in distilled water for at least 24 hr. Just prior to use, the Bio-Gel was washed and equilibrated with fresh perifusion medium. The perifusion chamber and a reservoir were kept in a water bath at 37°. The cells were perifused with the medium continuously bubbled with 100% O_2 at a flow rate of 1 mL/min. The cells were preperifused for 10 min before the start of each experiment. The perifusates were collected every 30 sec. After each perifusion, the cells in the perifusion chamber were collected and lysed by homogenizing in 20 mM potassium phosphate, pH 6.9, containing 6.7 mM NaCl and 0.1% bovine serum albumin. The lysate was centrifuged for 5 min at 900 *g*, and the supernatant was saved. Amylase activities in each fraction and in the supernatant were assayed by the method of Bernfeld [12] using soluble starch (Sigma) as the substrate. Total amylase activity remaining in the cell prior to each stimulation was calculated. The rate of amylase release was expressed as a percentage of the total activity.

Measurement of $[Ca^{2+}]_i$. Cells were incubated in 5 mL of a buffer, pH 7.4, consisting of the following: 127 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, and 0.1% bovine serum albumin with 2 μM fura-2 AM for 45 min at 37°, washed twice by alternating suspension and centrifugation, and resuspended in 1 mL of fresh buffer containing 1% bovine serum albumin. Just before use, 100-μL aliquots of cells were washed and resuspended in 2 mL of fresh medium at 37° in a plastic cuvette. The fura-2 fluorescence of these cells was recorded in a Hitachi 4010 spectrofluorometer equipped with a magnetic stirrer and thermostatted cuvette holder, with excitation at 340 and 380 nm and emission at 510 nm (the excitation wavelength was shifted between 340 and 380 nm at an interval of 4 sec). Addition to the cuvette was made in small volumes with no interruption in recording. Following each experiment, cells were lysed with 0.05% Triton X-100 to obtain the maximum fluorescence. This was followed by the addition of 2 mM [ethylenebis(oxyethylenenitrile)]-tetraacetic acid (EGTA) and 5 mM Tris buffer at pH 8.5 to determine minimum fluorescence. Background fluorescence was measured by adding 5 mM $MnCl_2$. Free Ca^{2+} was determined according to the method described by Grynkiewicz *et al.* [13] using a K_d of 224 nM for the fura-2–Ca^{2+} complex. The viability and carbamylcholine sensitivity of fura-2-loaded parotid acinar cells was almost the same as that of cells used for the determination of amylase release, as determined by carbamylcholine-stimulated amylase release (data not shown).

Materials. Fura-2 acetoxymethylester (fura-2 AM) was obtained from Molecular Probes, Inc. (Eugene, OR). (*l*)-Isoproterenol-D-bitartrate, 8-bromo adenosine 3′,5′-cyclic monophosphate (8-bromo cyclic AMP), carbamylcholine chloride, A23187, forskolin, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7), 3-isobutyl-1-methylxanthine (IBMX), PDBu, collagenase (type IA) and hyaluronidase were obtained from the Sigma Chemical Co. (St. Louis, MO). Ionomycin was obtained from Hoechst (Tokyo). Trifluoperazine dihydrochloride (Smith Kline & French Laboratories, Philadelphia, PA) was supplied as a gift. BAPTA-AM (1,2-bis [2-aminophenoxy] ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetomethyl ester) was obtained from the Dojindo Laboratories (Kumamoto). Atropine sulfate was obtained from Nakarai Chemicals, Ltd. (Kyoto). The maximum final concentration of dimethyl sulfoxide (0.1%), the vehicle for PDBu, A23187, ionomycin, BAPTA-AM and forskolin, had no effect on amylase secretion.

RESULTS

Potentiation of carbamylcholine-induced amylase secretion by isoproterenol. Figure 1 shows the time course of changes in the rate of amylase secretion in perifused rat parotid cells stimulated by 1-min pulses with carbamylcholine, isoproterenol and combinations of both. The mean resting (non-stimulated) rate of amylase secretion was about 0.15%/30 sec of the total amount of amylase remaining in the tissues prior to each stimulation. One micromolar carbamylcholine alone only slightly, but rapidly, increased the rate of amylase secretion with its peak being 30–60 sec after the onset of stimulation. Amylase secretion induced by 0.1 or 1 μM isoproterenol developed more slowly with the

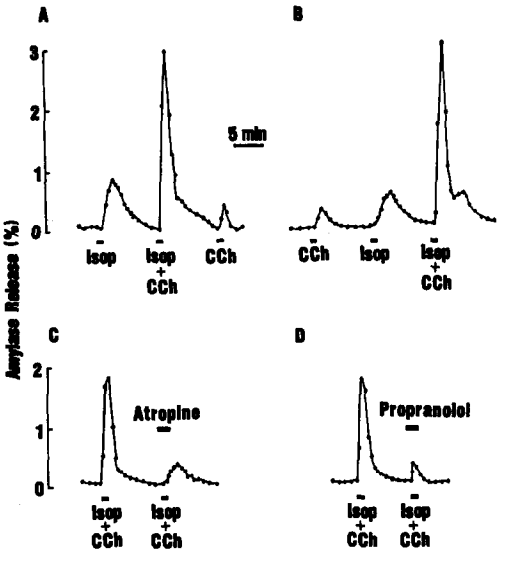

Fig. 1. Potentiation of amylase secretion induced by a combination of carbamylcholine (CCh) and isoproterenol (Isop). Perifused parotid acinar cells were stimulated by 1-min pulses with 0.1 μM (A) or 1 μM (B) isoproterenol, 1 μM carbamylcholine and combinations of both. Panels C and D show the effects of 1 μM atropine and 1 μM propranolol, respectively, on the potentiation of amylase secretion induced by 0.1 μM isoproterenol plus 1 μM carbamylcholine. The rate of amylase secretion was expressed as a percent per 30 sec of the total amylase activity remaining in tissues prior to each stimulation. Traces are representatives of at least three independent cell preparations.

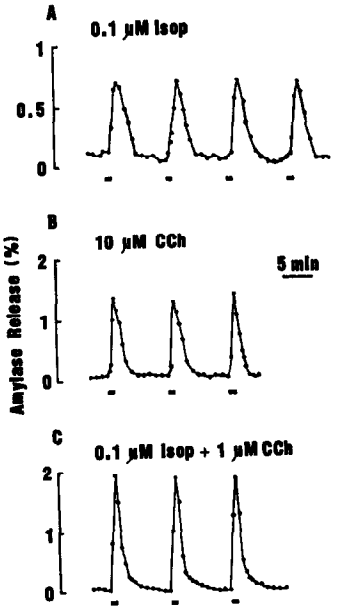

Fig. 2. Effect of repetitive stimulation on amylase secretion induced by various agonists. Perifused parotid cells were stimulated repeatedly by 1-min pulses with 0.1 μM isoproterenol (Isop) (A), 10 μM carbamylcholine (CCh) (B) and 0.1 μM isoproterenol plus 1 μM carbamylcholine (C). Traces are representatives of at least three independent cell preparations.

peak rate being at about 3 min. The time for the rate of amylase secretion to decay from the peak to half of the peak ($T_{1/2}$) was 131.0 ± 8.2 sec (N = 7) for 1 μM isoproterenol and 98.7 ± 4.0 sec (N = 6) for 0.1 μM isoproterenol. Combined additions of both 0.1 or 1 μM isoproterenol and 1 μM carbamylcholine induced a very rapid and large increase in the rate of amylase secretion with the peak being 60–90 sec after the onset of stimulation followed by a second peak or a hump. The maximum rate of amylase secretion attained with 0.1 or 1 μM isoproterenol plus 1 μM carbamylcholine was much higher than that using 1 μM isoproterenol alone. Concentration–response analysis of amylase secretion by isoproterenol showed that 1 μM isoproterenol gave almost the maximum stimulation of amylase secretion in our perifusion system (data not shown). In these experiments, the order of addition of agonists did not essentially change the results. The $T_{1/2}$ calculated by the initial rate of decay from the peak was 44.5 ± 3.0 sec (N = 6) for 0.1 μM isoproterenol plus 1 μM carbamylcholine and 40.6 ± 3.4 sec (N = 5) for 1 μM isoproterenol plus 1 μM carbamylcholine. These values were very similar to that for 10 μM [36.2 ± 2.2 sec (N = 4)] or 100 μM [39.3 ± 1.3 sec (N = 7)] carbamylcholine. The potentiation of amylase secretion between isoproterenol and carbamylcholine was inhibited completely by either the β-adrenergic antagonist propranolol or the muscarinic cholinergic antagonist atropine (Fig. 1).

Concentration–response of amylase secretion induced by carbamylcholine. Since repetitive 1-min pulse stimulations with 0.1 μM isoproterenol (Fig. 2A), 10 μM carbamylcholine (Fig. 2B) and 0.1 μM isoproterenol plus 1 μM carbamylcholine (Fig. 2C) were possible without decreases in the effects of these agonists, we next examined (Fig. 3) the concentration–response of amylase secretion with carbamylcholine in the presence and absence of 0.1 μM isoproterenol by repeating 1-min pulse stimulations with increasing concentrations of carbamylcholine. In the absence of isoproterenol it required about 1 μM carbamylcholine to cause significant stimulation of amylase secretion (Fig. 3A-b). Amylase secretion induced by carbamylcholine was increased by increasing the concentration of carbamylcholine from 1 to 100 μM. The peak rate of amylase secretion attained with 1 mM carbamylcholine was only slightly (about 5%) higher than that induced by 100 μM carbamylcholine. Previously we reported that repetitive 1-min pulse stimulations with 100 μM carbamylcholine cause the development of refractoriness [8]. The peak rate of amylase secretion attained by the second pulse stimulation with 100 μM carbamylcholine was slightly lower (about 85%) than that caused by the first one (data not shown). Therefore, the actual value of the peak rate of amylase secretion brought about by 1 mM carbamylcholine may be higher than that shown in this figure. In the presence of 0.1 μM

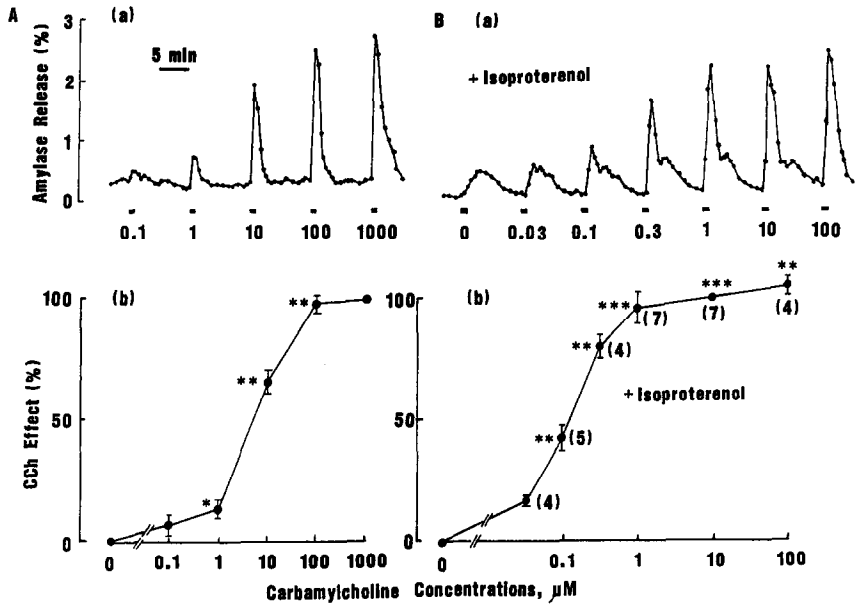

Fig. 3. Concentration–response of amylase secretion induced by carbamylcholine (CCh). Perifused parotid cells were stimulated by 1-min pulses with increasing concentrations of carbamylcholine in the absence (A) and presence (B) of isoproterenol. (a) Typical tracing. (b) Concentration–response. (A) The increase in the rate of amylase secretion induced by 1 mM carbamylcholine at 1 min after the onset of stimulation was taken as 100%. Each point is expressed as a percent of this value. Means of four independent cell preparations are shown with their standard errors. (B) The increase in the rate of amylase secretion induced by the combined addition of 10 μM carbamylcholine plus 0.1 μM isoproterenol above that by 0.1 μM isoproterenol alone at 90 sec after the onset of stimulation was taken as 100%. Each point is expressed as a percent of this value. Numbers in parentheses show the number of paired experiments. Means ± SEM are shown. Asterisks indicate the level of significance against the control as assessed by Student's *t*-test for paired samples: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$.

isoproterenol, 0.1 μM carbamylcholine significantly increased the rate of amylase secretion with the maximum effect being attained at about 1 μM carbamylcholine (Fig. 3B). Since the magnitude of the rate of amylase secretion induced by carbamylcholine stimulation alone or by the combined addition of carbamylcholine and isoproterenol varied markedly from preparation to preparation, the increases in the rates of amylase secretion induced by 1 mM carbamylcholine (Fig. 3A-b) and 10 μM carbamylcholine plus 0.1 μM isoproterenol (Fig. 3B-b), respectively, were taken as 100%. Each point shown in panels 3A-b and 3B-b is expressed as a percentage of these values. The half-maximum concentration for carbamylcholine to stimulate amylase secretion calculated from Eadie–Hofstee plots (apparent K_m) was 0.172 μM in the presence of, and 7.46 μM in the absence of 0.1 μM isoproterenol. As discussed above, there may be some uncertainty in the apparent K_m value calculated from the results obtained in the absence of isoproterenol, but these results show that isoproterenol increased the apparent affinity for carbamylcholine to stimulate amylase secretion. Since repetitive 1-min pulse stimulations with combined addition of 0.1 μM isoproterenol and 1 μM carbamylcholine were possible without changes in the peak rate of amylase secretion (Fig. 2), we compared the effect of 100 μM or 1 mM carbamylcholine with that of 0.1 μM isoproterenol plus 1 μM carbamylcholine. The peak rates of amylase secretion for 100 μM carbamylcholine and 1 mM carbamylcholine were 74.7 ± 4.0% (N = 3) and 75.6 ± 2.9% (N = 4) of that attained by the combined addition of 0.1 μM isoproterenol and 1 μM carbamylcholine, respectively (Fig. 4).

Potentiation of carbamylcholine-induced amylase secretion by 8-bromo cyclic AMP, IBMX, and forskolin. Two millimolar 8-bromo cyclic AMP, an analogue of cyclic AMP, and 0.1 mM IBMX or 5 μM forskolin only slightly increased the rate of amylase secretion (Fig. 5). Their effects on amylase secretion, like that of isoproterenol, developed very slowly with the peak rate being attained at about 3 min after the onset of stimulation. The effect of 1 μM carbamylcholine on amylase secretion was augmented markedly in the presence of 8-bromo cyclic AMP, IBMX and forskolin with the peak rate being about 60 sec after the onset of stimulation. The $T_{1/2}$ calculated from the initial rate of decay was 46.2 ± 4.8 sec (N = 4) for 1 μM carbamylcholine plus 2 mM 8-bromo cyclic AMP, 31.2 ± 3.6 sec (N = 4) for 1 μM carbamylcholine plus 0.1 mM IBMX, and 57.8 ± 6.9 sec (N = 5) for 1 μM carbamylcholine plus 5 μM forskolin. Again these values were very close to those using 10 or 100 μM carbamylcholine alone. The peak rate of amylase secretion induced by 1 μM carbamylcholine plus 100 μM IBMX or

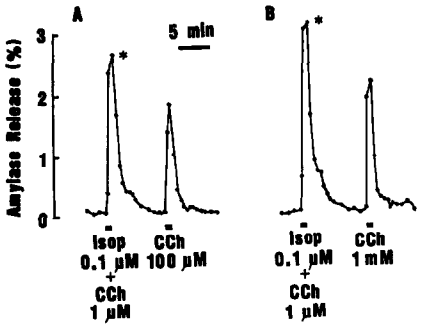

Fig. 4. Comparison of the peak rate of amylase secretion obtained by the combined addition of 1 μM carbamylcholine (CCh) plus 0.1 μM isoproterenol (Isop) with that induced by 100 μM or 1 mM carbamylcholine. Perifused parotid cells were first stimulated by 1-min pulses with 0.1 μM isoproterenol plus 1 μM carbamylcholine and then with 100 μM carbamylcholine (A) or 1 mM carbamylcholine (B). Traces are representatives of three independent cell preparations. An asterisk indicates the level of significance as assessed by Student's t-test for paired samples: (*) $P < 0.05$.

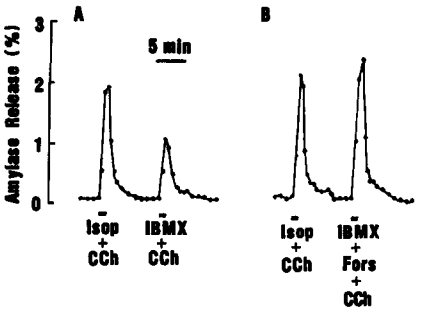

Fig. 6. Comparison of the rate of amylase secretion induced by 0.1 μM isoproterenol (Isop) plus 1 μM carbamylcholine (CCh) with that caused by either 100 μM IBMX plus 1 μM carbamylcholine or 100 μM IBMX plus 5 μM forskolin (Fors) plus 1 μM carbamylcholine. Perifused parotid cells were first stimulated by 1-min pulses with 0.1 μM isoproterenol plus 1 μM carbamylcholine and then with either 100 μM IBMX plus 1 μM carbamylcholine (A) or 100 μM IBMX plus 5 μM forskolin plus 1 μM carbamylcholine (B). Traces are representatives of at least three independent cell preparations.

5 μM forskolin, however, was lower than that brought about by 1 μM carbamylcholine plus 0.1 μM isoproterenol. This may be due to the smaller effect of IBMX or forskolin alone in increasing the amount of cyclic AMP, since the peak rate of amylase secretion induced by 1 μM carbamylcholine in the presence of 100 μM IBMX plus 5 μM forskolin, a condition which markedly increased the level of cyclic AMP (data not shown), was almost the same as that induced by 0.1 μM isoproterenol plus 1 μM carbamylcholine (Fig. 6).

Calcium involvement in the potentiation. In our perifusion system, 5 μM A23187 or 5 μM ionomycin,

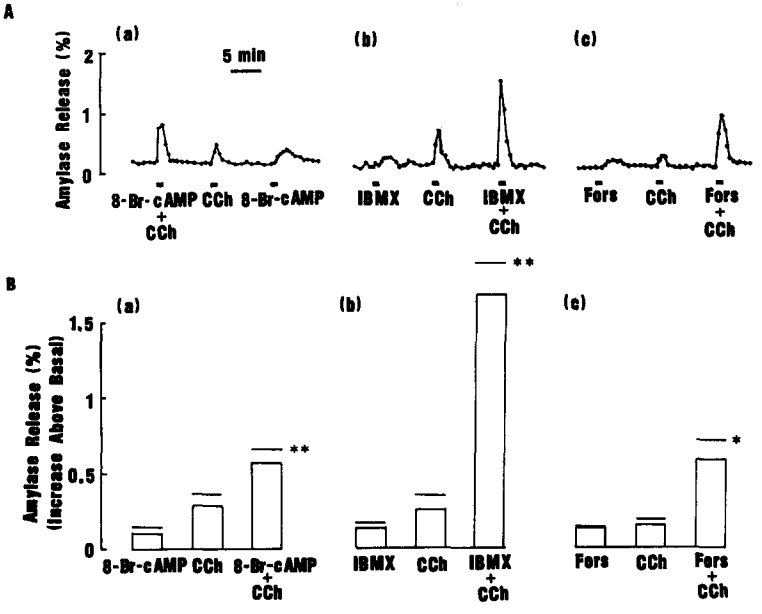

Fig. 5. Potentiation of 1 μM carbamylcholine (CCh)-induced amylase secretion by 2 mM 8-bromo cyclic AMP (8-Br-cAMP) (a), 0.1 mM IBMX (b) and 5 μM forskolin (Fors) (c). Perifused parotid cells were stimulated by 1-min pulses with various agonists as indicated in the figures. (A) Each trace is representative of four independent cell preparations. (B) The peak rates of amylase secretion obtained with each treatment are shown with their standard errors. Asterisks, indicate the level of significance as assessed by Student's t-test for paired samples: (*) $P < 0.05$; (**) $P < 0.01$.

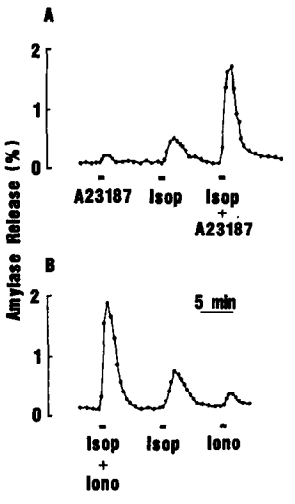

Fig. 7. Potentiation of calcium ionophore-induced amylase secretion by isoproterenol (Isop). Perifused parotid cells were stimulated by 1-min pulses with either 0.1 μM isoproterenol, 5 μM A23187 and a combination of both (A) or 0.1 μM isoproterenol, 5 μM ionomycin (Iono) and a combination of both (B). Traces are representatives of at least three (both A and B) independent cell preparations.

which alone had only a small effect, markedly increased the rate of amylase secretion in the presence of isoproterenol (Fig. 7). The $T_{1/2}$ calculated in the presence of isoproterenol was 51.2 ± 3.8 sec (N = 3) for A23187 and 55.8 ± 4.0 sec (N = 3) for ionomycin, and again these values were very close to those obtained using 10 or 100 μM carbamylcholine, but not to that induced by isoproterenol.

The peak rate of amylase secretion attained with the first stimulation using either 1 μM carbamylcholine plus 0.1 or 1 μM isoproterenol in calcium-free medium (calcium omission plus 0.2 mM EGTA) was not different from that obtained in the presence of calcium, but amylase secretion induced by 10 μM carbamylcholine markedly decreased after the removal of calcium from the perifusion fluid (Fig. 8A). The peak rate of amylase secretion with all the agonists in calcium-free medium gradually decreased as the stimulation was repeated. Therefore, we did not further examine the effect of calcium omission on the potentiation.

The contribution of calcium ions to the potentiation was also examined by loading the cells with BAPTA, an intracellular calcium chelator. By preexposing the cells to 20 μM BAPTA-AM for 10 min, amylase secretion induced by 0.1 μM isoproterenol plus 1 μM carbamylcholine (Fig. 8B-c) and 10 μM carbamylcholine (Fig. 8B-a) was decreased markedly, and the time course of changes in the rate of amylase secretion induced by 0.1 μM isoproterenol plus 1 μM carbamylcholine became very similar to that caused by 0.1 μM isoproterenol alone with the $T_{1/2}$ being 80.1 ± 9.8 sec (N = 4). On the other hand, amylase secretion induced by isoproterenol was affected only a little (Fig. 8B-b). The peak rate of amylase secretion induced by isoproterenol was attained at about 3 min after the onset of stimulation both in the presence and absence of BAPTA-AM.

Effect of PDBu. PDBu (1 μM), which alone had only a slight effect, markedly potentiated amylase secretion induced by isoproterenol (Fig. 9A). Since repetitive stimulations with 0.1 μM isoproterenol plus 0.1 μM PDBu were possible without changes in both the time course and the peak level of amylase secretion (Fig. 9B), we examined the concentration–response of amylase secretion induced by PDBu in the presence of 0.1 μM isoproterenol. The effect of PDBu was significant at 0.01 μM PDBu with the maximum effect being obtained at 1 μM (Fig. 9C). However, the time course of changes in amylase secretion induced by isoproterenol plus PDBu was essentially the same as that due to isoproterenol alone. The peak rate of amylase secretion induced by the combined addition of 0.1 μM isoproterenol plus 0.1 μM PDBu was attained at about 3 min after the onset of stimulation with $T_{1/2}$ being 141.0 ± 4.2 sec (N = 4). Amylase secretion induced by the combined addition of 0.1 μM PDBu and 5 μM A23187 was 0.279 ± 0.014 % (N = 4) which was much smaller than that by 10 μM carbamylcholine.

Effect of calmodulin antagonists. The calmodulin antagonist W-7 (200 μM) (Fig. 10) inhibited amylase secretion induced by 1 μM carbamylcholine plus 0.1 μM (Fig. 10A-a) or 1 μM (data not shown) isoproterenol. Slight inhibition was observed at 50 μM W-7. The inhibitory effect of W-7 was further increased by increasing the concentrations of W-7. It was about 70% at 500 μM W-7. We did not use higher concentrations of W-7 because W-7 by itself stimulated amylase secretion (data not shown). W-7 also inhibited the effect of 10 μM carbamylcholine (Fig. 10A-b) and 10 μM norepinephrine (Fig. 10A-d), but not that of 1 μM isoproterenol (Fig. 10A-c). As shown in this figure, the time course of changes in the rate of amylase secretion induced by 1 min pulse stimulations with 10 μM norepinephrine was very similar to that by isoproterenol plus carbamylcholine, but not to that by isoproterenol alone, with the $T_{1/2}$ being 38.5 ± 2.5 sec (N = 6) (Fig. 10A-d). Amylase secretion induced by norepinephrine was not changed by repeating the stimulation, but was decreased markedly by both α- and β-adrenergic antagonists (data not shown). The time course of changes in the rate of amylase secretion induced by norepinephrine in the presence of prazosin was similar to that induced by isoproterenol alone. Amylase secretion by norepinephrine in the presence of propranolol became very small and transient. W-7 also slightly decreased the peak rate of amylase secretion induced by the combined addition of 0.1 μM isoproterenol plus 5 μM A23187 (Fig. 10A-e), but its effect was not statistically significant. Amylase secretion induced by 1-min pulse stimulation with 0.1 μM isoproterenol plus 5 μM A23187 did not change by repeating the stimulation. In the presence of 200 μM W-7, however, the time to reach the peak slightly, but consistently, slowed and the recovery from its peak was delayed markedly. Similar results were obtained using

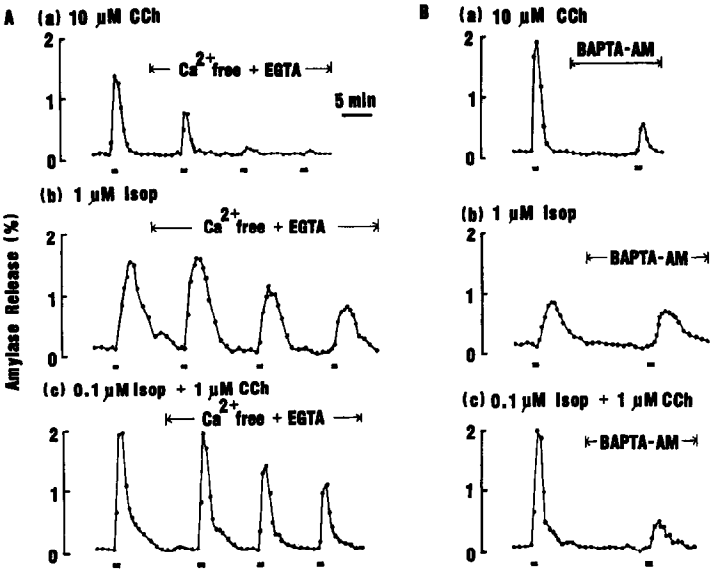

Fig. 8. Role of calcium in the potentiation of amylase secretion. (A) Perifused parotid cells were first stimulated by a 1-min pulse with the agonists indicated. They were then preperifused for 10 min in calcium-free (Ca^{2+} free + 0.2 mM EGTA) fluid, and stimulated three times by 1-min pulses with the same agonists. (B) Perifused parotid cells were first stimulated by 1-min pulses with the agonists indicated. They were preperifused with 20 μM BAPTA-AM for 10 min and then stimulated again with the same agonists. Traces are representatives of at least three (both A and B) independent cell preparations. Abbreviations: CCh, carbamylcholine, and Isop, isoproterenol.

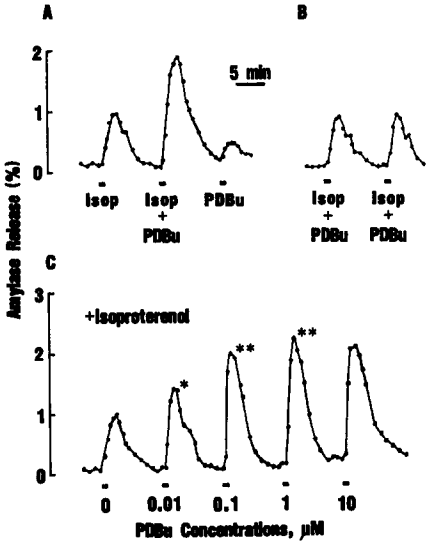

Fig. 9. Potentiation of amylase secretion induced by the combined addition of isoproterenol (Isop) and PDBu. (A) Perifused parotid cells were stimulated by 1-min pulses with 0.1 μM isoproterenol, 1 μM PDBu and combinations of both. (B) Repetitive stimulations with 0.1 μM isoproterenol plus 0.1 μM PDBu. (C) Concentration–response of amylase secretion induced by PDBu in the presence of 0.1 μM isoproterenol. Traces are representatives of at least three (A, B and C) independent cell preparations. Asterisks indicate the level of significance of the effect of PDBu as assessed by Student's *t*-test for paired samples: (*) $P < 0.05$; and (**) $P < 0.01$.

another calmodulin antagonist trifluoperazine (Fig. 11).

Effect of isoproterenol and carbamylcholine on $[Ca^{2+}]_i$. Figure 12 shows the effect of carbamylcholine on $[Ca^{2+}]_i$ in fura-2-loaded parotid acinar cells in batch systems. The higher concentrations of carbamylcholine (10 and 100 μM) evoked a rapid, but transient, initial peak in $[Ca^{2+}]_i$ at about 20 sec after the onset of stimulation, which was followed by a lower sustained phase (Fig. 12). One micromolar carbamylcholine did not raise the initial peak in $[Ca^{2+}]_i$, but slowly increased $[Ca^{2+}]_i$ by 82.5 ± 6.8 nM (N = 6) at 20 sec after the onset of stimulation. Isoproterenol (0.1 μM) alone did not increase $[Ca^{2+}]_i$ significantly and did not augment the effect of 1 μM carbamylcholine. The increase in $[Ca^{2+}]_i$ induced by the combined addition of 0.1 μM isoproterenol and 1 μM carbamylcholine was 87.9 ± 14.5 nM.

DISCUSSION

Previously we have shown that perifusion of parotid acinar cells is a useful system for examining the dynamic changes in amylase secretion induced by various secretagogues, since in this system the time course of changes in amylase secretion induced by various agonists can be followed [8]. We used this system to study the mechanism of potentiation of amylase secretion between carbamylcholine and isoproterenol. In the present experiment we used 1-

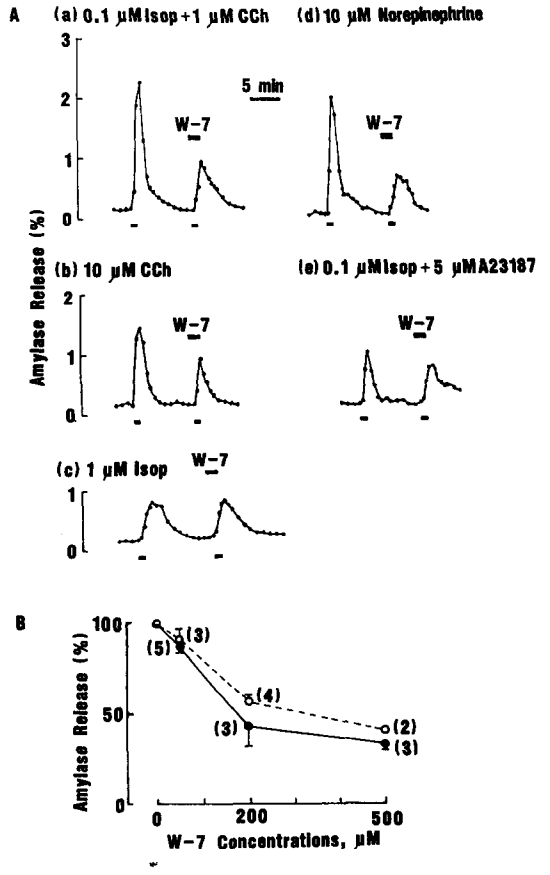

Fig. 10. Inhibition of agonist-induced amylase secretion by 200 μM W-7. Perifused parotid cells were stimulated by 1-min pulses with various agonists in the presence and absence of W-7. Addition of W-7 was started 1 min prior to the onset of stimulation with the agonists. (A) Typical tracings. (B) Concentration–response of inhibition by W-7. The peak responses obtained in the absence of W-7 at 90 sec for 0.1 μM isoproterenol (Isop) plus 1 μM carbamylcholine (CCh) (●——●) and at 60 sec for 10 μM carbamylcholine (○——○) after the onset of stimulation were taken as 100%. Each value obtained in the presence of W-7 is expressed as a percent of the peak response. Means ± SEM are shown. Numbers in parentheses show the number of paired experiments.

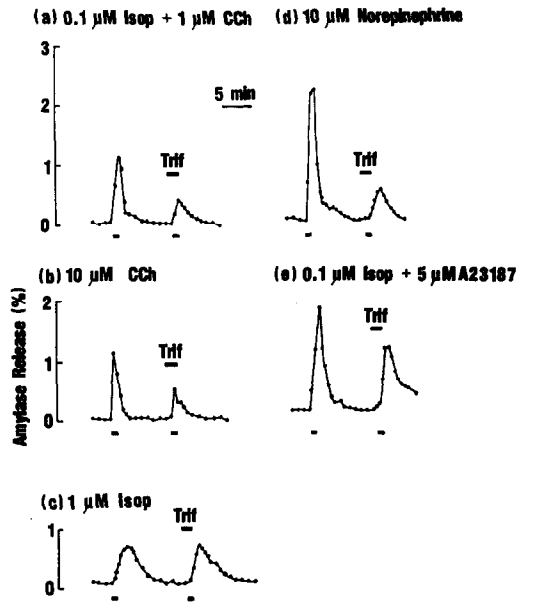

Fig. 11. Inhibition of agonist-induced amylase secretion by 200 μM trifluoperazine (Trif). The conditions of the experiments are the same as those shown for Fig. 10. Traces are representatives of at least three independent cell preparations.

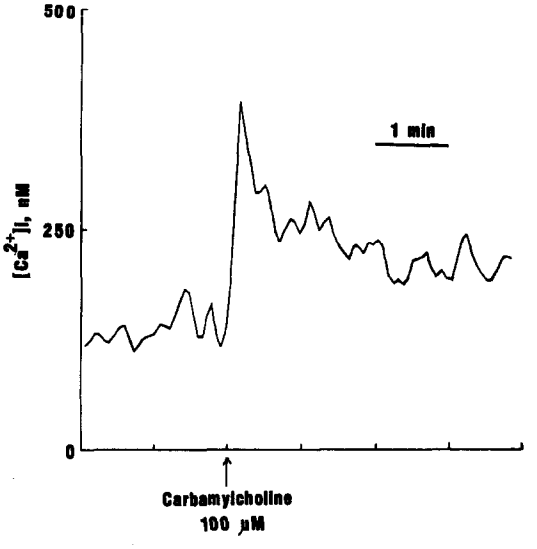

Fig. 12. Effect of carbamylcholine on $[Ca^{2+}]_i$. Fura-2-loaded parotid acinar cells were stimulated with 100 μM carbamylcholine. Trace is representative of at least six independent cell preparations.

min pulse stimulations because it may minimize the influence of rapid development of desensitization to carbamylcholine [8]. The findings of the present study suggest that the potentiation is due to the enhancement of carbamylcholine-induced amylase secretion by isoproterenol. This contention is based on the following two observations: First, the time course of changes in the rate of amylase secretion induced by the combined additions of low concentrations of carbamylcholine and isoproterenol was very similar to that induced by higher concentrations of carbamylcholine alone, but not to that brought about by isoproterenol. Second, calmodulin antagonists inhibited amylase secretion induced by carbamylcholine and carbamylcholine plus isoproterenol, but not that caused by isoproterenol.

The effect of isoproterenol on the potentiation can be mimicked with a cyclic AMP analogue and agonists which raise the parotid cyclic AMP

level. Thus, 8-bromo cyclic AMP potentiated carbamylcholine-induced amylase secretion. Forskolin, a direct activator of adenylate cyclase [14], and IBMX, which increases the level of parotid cyclic AMP by inhibiting the degradation of cyclic AMP [15], also mimicked the effect of isoproterenol. The existence of a common effect of these diverse agents suggests a central role for cyclic AMP in the mediation of the potentiation. It has been reported that 1 μM carbamylcholine fails to elicit a significant change in isoproterenol-induced cyclic AMP accumulation [9]. These results suggest that the potentiation of carbamylcholine-induced amylase secretion by isoproterenol is mediated by cyclic AMP and that it results from the action at a step distal to the generation of cyclic AMP.

Carbamylcholine causes the activation of phospholipase C, which results in the formation of IP_3 and diacylglycerol [1, 16, 17]. IP_3 increases $[Ca^{2+}]_i$ by stimulating the release of calcium from intracellular stores [5, 6]. Carbamylcholine also increases $[Ca^{2+}]_i$ by increasing the influx of Ca^{2+} from extracellular fluid [18]. It has been reported that the effect of carbamylcholine on amylase secretion is decreased markedly by calcium omission from extracellular fluid [7, 19]. Because calcium seems to be important in the mechanism of amylase secretion induced by carbamylcholine, its involvement was investigated as a necessary factor in the potentiation. To this end we first used calcium ionophores (A23187 and ionomycin) and found the potentiation of amylase secretion between the calcium ionophores and isoproterenol with the time course of the changes in amylase secretion being very similar to that caused by carbamylcholine plus isoproterenol. Calcium omission from the perifusion fluid did not decrease the effect of the first pulse stimulation with isoproterenol plus 1 μM carbamylcholine, but it markedly decreased that with 10 μM carbamylcholine. This result shows that the extracellular calcium is not directly involved in the potentiation, but is important in the expression of the maximum effect of carbamylcholine. The gradual decrease in the rate of amylase secretion due to repetitive stimulations in calcium-free medium suggests that the amount of calcium released from the intracellular calcium store may be a determining factor in the regulation of amylase secretion, that is, the gradual decline may be associated with the degree of depletion of calcium content in the intracellular calcium store. The degree of the decrease in the rate of amylase secretion induced by carbamylcholine after repetitive stimulations was larger than that with isoproterenol plus 1 μM carbamylcholine. This may be consistent with our results showing that, in the presence of isoproterenol, only a small increase in $[Ca^{2+}]_i$ induced by lower concentrations of carbamylcholine was sufficient for a large increase in the rate of amylase secretion. Another way to examine the role of calcium in the potentiation is to load the cells with BAPTA, an intracellular calcium chelator [20]. Loading the parotid cells with BAPTA substantially inhibits the increase in $[Ca^{2+}]_i$ caused by substance P and ionomycin [21]. In our results preperifusion with BAPTA-AM for 10 min almost completely abolished the potentiation of amylase secretion induced by the combined addition of isoproterenol and carbamylcholine. These results suggest that calcium released from the intracellular store directly participates in this potentiation.

Diacylglycerol, another endogenous product of phospholipase C, is suggested to modulate physiological responses by activating protein kinase C [17]. Stimulation of amylase secretion by phorbol esters or synthetic diacylglycerol derivatives [22], the endogenous activator of protein kinase C, provides major evidence implicating protein kinase C in the mechanism of amylase secretion. McKinney and Rubin [11] reported that phorbol dibutyrate potentiates amylase secretion induced by isoproterenol, forskolin and dibutyryl-cyclic AMP, and suggested that the potentiation is mediated by interaction between protein kinase C and cyclic AMP. We observed similar results by using rat parotid slices in batch systems [23]. Protein kinase C is translocated from the cytosol to the membrane, and the enhanced association of the enzyme with the membrane is suggested to lead to its activation [17, 24, 25]. Machado-De Domenech and Söling [26], by using guinea pig parotid cells, reported that the activation of muscarinic cholinergic receptors results in redistribution of cytosolic protein kinase C to particulate fractions. In our results obtained in rat parotid slices, however, we did not find the redistribution of protein kinase C after stimulation by carbamylcholine, although we observed this with the use of PDBu [27]. We also observed the potentiation of carbamylcholine-induced amylase secretion by PDBu [27]. Thus, we could not obtain any evidence suggesting the participation of protein kinase C in amylase secretion induced by carbamylcholine in rat parotid slices. In our perifused parotid acinar cells we confirmed the potentiation of amylase secretion by a combination of isoproterenol and PDBu, but the time course of changes in the rate of amylase secretion induced by isoproterenol plus PDBu was different from that brought about by a combination of isoproterenol and carbamylcholine. Thus, PDBu cannot mimick the effect of carbamylcholine on enhancing the rate at which the maximum release is reached. Therefore, although the activation of protein kinase C by phorbol dibutyrate may potentiate amylase secretion induced by isoproterenol, protein kinase C may not participate in the potentiation when carbamylcholine and isoproterenol were used together.

All of these results suggest that the potentiation of amylase secretion induced by the combined addition of carbamylcholine and isoproterenol is mainly, if not completely, a post-receptor event that occurs at a step distal from the second messenger (cyclic AMP and Ca^{2+}) generation and that the potentiation is caused by a cyclic AMP modulation of the calcium messenger system. We do not know how the increase in $[Ca^{2+}]_i$ participates in the regulation of amylase secretion. Amylase secretion induced by carbamylcholine and isoproterenol plus carbamylcholine was inhibited by calmodulin antagonists such as W-7 and trifluoperazine. Similarly, amylase secretion induced by norepinephrine, the effect of which is caused by synergistic interaction between α- and β-adrenergic receptor

activation [28], was inhibited markedly by calmodulin antagonists. These results tempted us to think that calmodulin may participate in a calcium-regulated secretory mechanism. However, W-7 and chlorpromazine are reported to act as muscarinic [29, 30] and α-adrenergic [31] antagonists. Therefore, the inhibition of (isoproterenol plus carbamylcholine)- and norepinephrine-induced amylase secretion by calmodulin antagonists could also occur through such activities. Amylase secretion induced by a combination of isoproterenol and A23187, however, was not inhibited significantly by calmodulin antagonists. These results are not in accord with the interpretation based on the involvement of calmodulin in the calcium-regulated secretory mechanism. However, the peak level of amylase secretion attained by a combination of isoproterenol and A23187 was much lower than those induced by isoproterenol plus carbamylcholine or norepinephrine. Furthermore, amylase secretion induced by isoproterenol plus A23187 in the presence of calmodulin antagonists decreased in both the times to reach the peak and to decay from the peak. These results may explain our failure to detect the inhibitory effect of calmodulin antagonists on amylase secretion induced by the combined addition of isoproterenol and A23187.

Our results are not in accord with those of McKinney *et al.* [28], who reported that synergism of amylase secretion between carbamylcholine and isoproterenol resides in enhanced calcium availability. They reported that 1 μM isoproterenol, which does not elevate $[Ca^{2+}]_i$, causes a small enhancement in the effect of carbamylcholine on $[Ca^{2+}]_i$. They also reported that dibutyryl-cyclic AMP alone fails to elevate $[Ca^{2+}]_i$ above the resting level, but causes a significant augmentation in $[Ca^{2+}]_i$ evoked by ionomycin. However, in our results, we could not observe any augmentation in $[Ca^{2+}]_i$ by a combination of carbamylcholine and isoproterenol. In our apparatus used to measure $[Ca^{2+}]_i$, it required 4 sec to shift the excitation wavelength between 340 and 380 nm. Tojyo *et al.* [32] have reported noticeable vibrations of fura-2 fluorescence when they used parotid acini prepared by the method essentially similar to our present experiments. Therefore, the discrepancy between our results and those of McKinney *et al.* may be due to our failure to follow the small and transient changes in $[Ca^{2+}]_i$. In our results, the peak rate of amylase secretion induced by low concentrations of carbamylcholine and isoproterenol, which only slightly increased $[Ca^{2+}]_i$, was significantly higher than that induced by 100 μM or 1 mM carbamylcholine alone which evoked a large increase in $[Ca^{2+}]_i$. These results suggest that the potentiation of amylase secretion induced by carbamylcholine plus isoproterenol cannot be adequately explained by assuming enhanced calcium availability only.

Concentration–response analysis of amylase secretion induced by carbamylcholine showed that isoproterenol increased the apparent affinity for carbamylcholine to stimulate amylase secretion. As discussed above, the potentiation induced by a combination of carbamylcholine and isoproterenol seems to be mainly, if not completely, caused by a cyclic AMP-modulation of a calcium-regulated process leading to amylase secretion. Thus, the potentiation of carbamylcholine-induced amylase secretion by isoproterenol is probably caused by a cyclic AMP-induced increase in the sensitivity of the calcium response to calcium. The mechanism by which cyclic AMP causes its modulatory effect is not known, but similar regulation of the calcium messenger system by cyclic AMP is reported in the activation of phosphorylase *b* kinase and myosin light chain kinase, which are both calcium-regulated enzymes [33, 34]. Phosphorylation of these proteins by cyclic AMP-dependent protein kinase leads to an alteration in their sensitivity to calcium [35]. Therefore, the potentiation of carbamylcholine-induced amylase secretion by cyclic AMP may be mediated by phosphorylation of the calcium response element via the activation of cyclic AMP-dependent protein kinase.

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