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Characterization of the Ca^{2+} Response Mediated by Activation of β-Adrenoceptors in Rat Submandibular Ducts

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ABSTRACT—The Ca^{2+} signaling mediated by activation of β-adrenoceptors was studied in a purified preparation of ducts from rat submandibular glands. At concentrations above 1 nM, isoproterenol (ISO) caused a small but significant increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The ISO-induced increase in $[Ca^{2+}]_i$ was completely inhibited by the β-adrenoceptor antagonist propranolol but not by the α-adrenoceptor antagonist phentolamine. Forskolin was able to mimic the Ca^{2+} response to ISO. These results suggest that the ISO-induced increase in $[Ca^{2+}]_i$ in rat submandibular ducts is mediated by an accumulation of cAMP resulting from activation of β-adrenoceptors. In the absence of extracellular Ca^{2+}, ISO or forskolin caused a transient increase in $[Ca^{2+}]_i$, indicating Ca^{2+} mobilization from intracellular Ca^{2+} stores. Further, stimulation with ISO failed to mobilize Ca^{2+} after the depletion of intracellular Ca^{2+} stores by phenylephrine or carbachol, suggesting that the cAMP-mediated increase in $[Ca^{2+}]_i$ is due to a Ca^{2+} release from inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores. As ISO did not stimulate a detectable production of IP_3, the cAMP-mediated Ca^{2+} mobilization may be evoked by a mechanism different from activation of phosphoinositide hydrolysis.

Keywords: Submandibular duct, β-Adrenoceptor, Cytosolic Ca^{2+}, Isoproterenol, Cyclic AMP

Saliva is initially produced as an isotonic primary secretion in the salivary acini. The primary fluid is modified by ductal reabsorption of sodium chloride and secretion of potassium and bicarbonate as the fluid flows through the salivary ducts (1). The saliva is finally discharged as a hypotonic fluid into the oral cavity. The proteins in saliva are mainly synthesized and secreted by acinar cells, but some proteins, including kallikrein and several growth factors, are known to be secreted by ductal cells (1, 2). Salivary ducts are thought to be innervated by both sympathetic and parasympathetic fibers, and there is abundant evidence that the ductal transport of ions and the protein secretion are under autonomic control.

Since the 1980s, monitoring of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in salivary gland cells has been extensively reported using enzymatically dispersed cells. Studies have demonstrated that the increase in $[Ca^{2+}]_i$ in salivary glands is induced by activation of muscarinic receptors and α-adrenoceptors but not by β-adrenoceptors. Most of the cells obtained by enzyme digestion are acinar cells (for rat parotid cells, approximately 95%) (3), indicating that the findings for Ca^{2+} signaling obtained using dispersed cells exclusively reflect the Ca^{2+} response of acinar cells. Due to the technical difficulties in cell isolation, there is much less information on Ca^{2+} signaling in ductal cells than those in acinar cells.

Dehaye et al. (4, 5) recently presented an efficient method for isolating a highly purified preparation of ducts from rat submandibular gland and reported that β-adrenergic stimulation resulted in Ca^{2+} mobilization from intracellular Ca^{2+} stores in the ductal cells. This response is inconsistent with that of acinar cells, in which activation of β-adrenergic receptors does not cause any change in $[Ca^{2+}]_i$ (6 – 8). The present study examined the effects of the β-adrenoceptor agonist isoproterenol (ISO) on $[Ca^{2+}]_i$ in ductal cells prepared from rat submandibular glands and further assessed the mechanism of Ca^{2+} mobilization induced by β-adrenergic stimulation.

MATERIALS AND METHODS

Materials

ISO, phenylephrine, carbachol, forskolin, propranolol, dibutyryl cyclic AMP (DBcAMP) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Collagenase P was from Boehringer Mannheim (Darmstadt, Germany). The *myo*-[2-^{3}H]-inositol was purchased from Muromachi Kagaku Kogyo (Tokyo). Fura-2

acetoxymethyl ester (fura-2/AM) and HEPES were from Dojin Laboratories (Kumamoto). Phentolamine was from Nippon Ciba-Geigy (Kawanishi). All other reagents were of analytical grade.

Preparation of submandibular ducts

Male Wistar-strain rats, weighing 200 – 250 g, were anesthetized with diethyl ether and killed by cardiac puncture. Submandibular ductal cells were prepared by the modified method of Dehaye and Turner (4). Briefly, four submandibular glands removed from two rats were finely minced and then incubated for 20 min at 37°C in 10 ml of Dulbecco's modified eagle medium (D-MEM) containing collagenase P (0.24 mg/ml), 2 mM L-glutamine and 0.2% BSA. After dispersion, the cell suspension was passed through a nylon mesh, and the filtrate was centrifuged ($400 \times g$ for 5 s). The resulting pellet was resuspended in 20 ml of fresh Hanks' balanced salt solution buffered with 20 mM HEPES-NaOH, pH 7.4 (HBSS-H) and washed by 1-min centrifugation at $100 \times g$. The cells were then washed again and finally resuspend in 6 ml of fresh HBSS-H.

Two 6-ml aliquots of an isotonic 40% Percoll solution were placed in 15-ml plastic tubes. Three milliliters of the cell suspension were layered on the top of the Percoll solution, and the tubes were centrifuged at $4{,}000 \times g$ for 10 min. After centrifugation, the cells were separated into two distinct populations. A cell population consisting predominantly of ducts remained in the interphase of the tube, while the acini sedimented to the bottom. These two cell populations were collected and washed twice and then resuspended in fresh HBSS-H. Figure 1 shows a bright-field image of the duct preparation obtained by this method.

The viability of the ductal cells was assessed by trypan blue exclusion. Their viability was estimated to be above 95% even after the Percoll centrifugation.

Measurement of $[Ca^{2+}]_i$

The dispersed cells were incubated for 45 min at 37°C in HBSS-H containing 2 μM fura-2/AM. The fura-2-loaded cells were washed twice and resuspended in fresh HBSS-H and then transferred into quartz cuvettes. The cuvettes were thermostatically controlled at 37°C, and the cell suspension was continuously stirred. Fura-2 fluorescence was measured with a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. At the end of each experiment, the maximum fluorescence was determined by lysing the cells with 0.1% Triton X-100, and minimum fluorescence was determined by the addition of 30 mM Tris base and 5 mM EGTA. The $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence, as described by Grynkiewicz et al. (9).

Measurement of inositol phosphates

Ductal cells were prelabelled with *myo*-[2-^{3}H]-inositol,

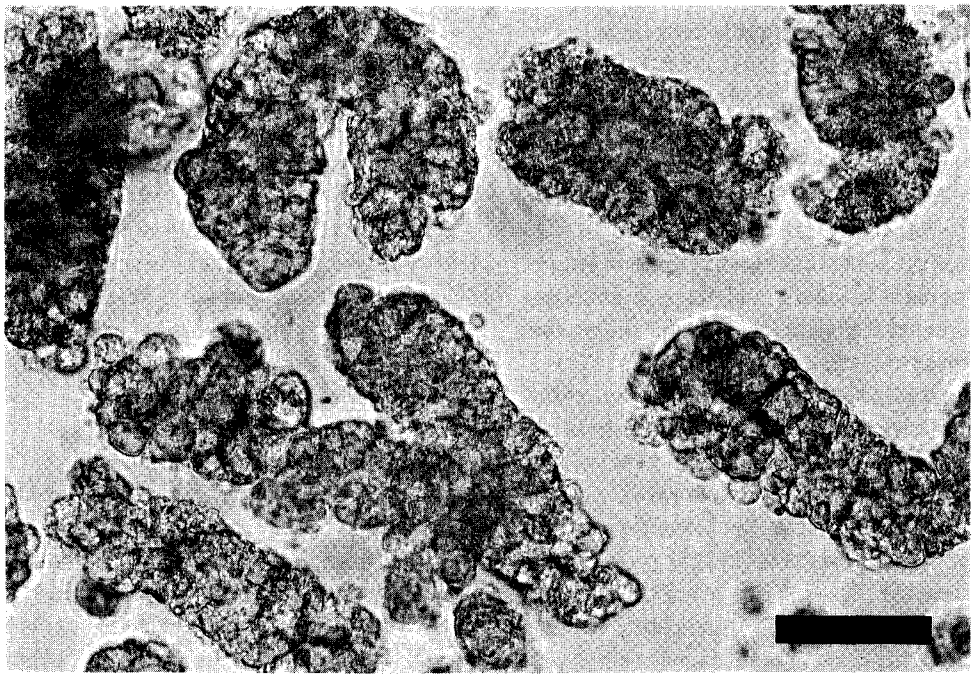

Fig. 1. Light micrograph of purified submandibular ducts. The scale bar indicates 100 μM.

as described elsewhere (10). The cells were preincubated for 5 min in the presence of 10 mM LiCl and then stimulated by addition of 1 μM ISO or 1 μM phenylephrine for a further 10 min at 37°C. The reactions were stopped by the addition of $HClO_4$ (final concentration of 4.5%). A portion of the extract was then neutralized with 0.5 M KOH / 9 mM $Na_2B_4O_7$. Labeled inositol trisphosphate was separated from other inositol phosphates on a Bio-Rad AG 1-X8 column according to the method of Berridge et al. (11).

Statistical analyses

The statistical significance of differences between values was determined by Student's *t*-test.

RESULTS

The effect of 1 μM ISO on $[Ca^{2+}]_i$ was examined in fura-2-loaded acini and ducts (Fig. 2A). Stimulation with ISO had no effect on the $[Ca^{2+}]_i$ in acini, whereas it caused a significant increase in $[Ca^{2+}]_i$ in ducts. The increase in $[Ca^{2+}]_i$ measured 10 s after addition of ISO was 58 ± 5 nM above the basal level (mean ± S.E.M., n = 7; $P<0.01$, compared with the basal). To show that the acini had maintained the ability to respond to stimuli for Ca^{2+} mobilization, the acini were stimulated with 1 μM phenylephrine, an α-adrenoceptor agonist, after stimulation with ISO. The stimulation with phenylephrine caused a significant increase in $[Ca^{2+}]_i$ in the acini, and the extent of the response

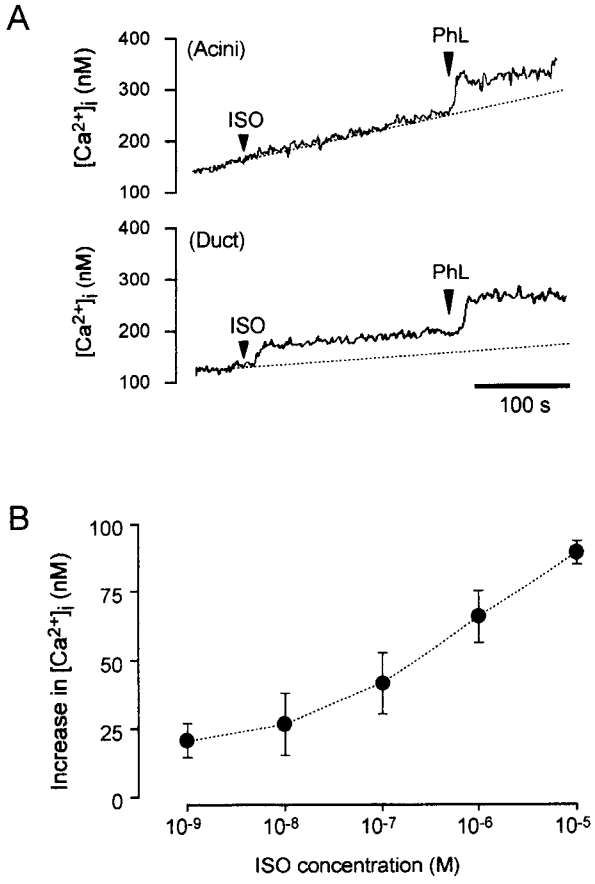

Fig. 2. Effects of isoproterenol (ISO) and phenylephrine (PhL) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in submandibular ducts and acini. A: Acini and ducts were stimulated with 1 μM ISO and then with 1 μM PhL in the presence of extracellular Ca^{2+}. The agonists were added to acini and ducts as indicated by arrowheads. The dotted lines represent apparent changes in $[Ca^{2+}]_i$, probably due to a slow leakage of fura-2, in unstimulated acini and ducts. B: Concentration-response relationship for the ISO-induced increase in $[Ca^{2+}]_i$ in ducts. The results show the increases in $[Ca^{2+}]_i$ above the basal level measured 10 s after stimulation with various concentrations of ISO. Values are means ± S.E.M. of 7 experiments.

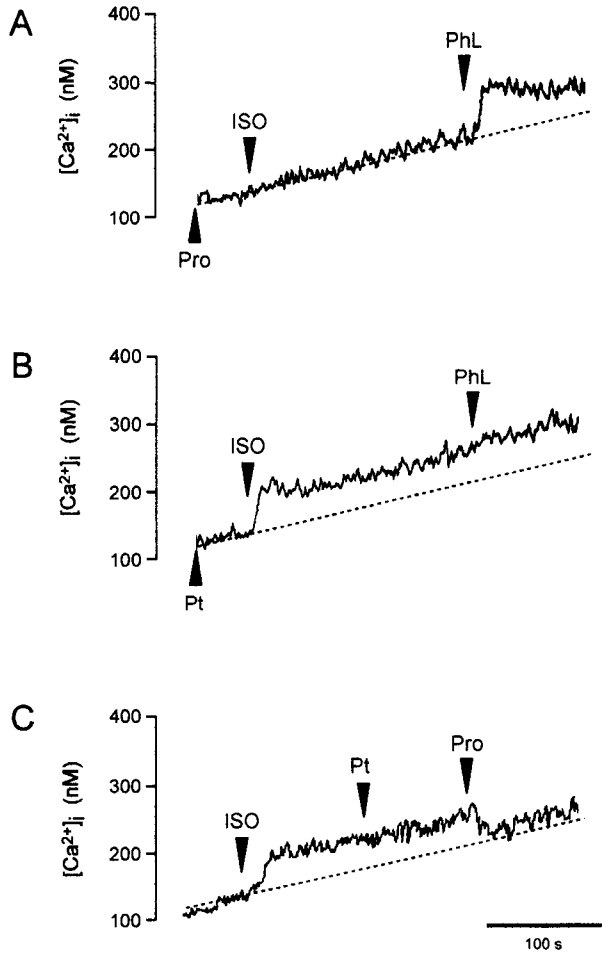

Fig. 3. Effects of propranolol (Pro) and phentolamine (Pt) on the ISO-induced increase in $[Ca^{2+}]_i$ in submandibular ducts. A: In the presence of 0.1 μM Pro, ducts were stimulated with 1 μM ISO and then with 1 μM PhL. B: In the presence of 0.1 μM Pt, ducts were stimulated with 1 μM ISO and then with 1 μM PhL. C: Following stimulation with 1 μM ISO, 0.1 μM Pt and 0.1 μM Pro were added as indicated by arrowheads. The dotted lines represent apparent changes in $[Ca^{2+}]_i$ in unstimulated ducts. The results are representative of 6 experiments.

was similar to that observed in ducts. Figure 2B shows the concentration-response relationship for the ISO-induced increase in $[Ca^{2+}]_i$ in ducts. The ducts were stimulated with various concentrations of ISO, and the $[Ca^{2+}]_i$ was measured 10 s after stimulation. At concentrations above 1 nM, ISO increased the $[Ca^{2+}]_i$ in a concentration-dependent manner.

To show that the ISO-induced increases in $[Ca^{2+}]_i$ in ductal cells resulted from activation of β-adrenoceptors, the ducts were preincubated with an α- or β-adrenoceptor antagonist and then stimulated with 1 μM ISO. Preincubation with 0.1 μM propranolol, a β-adrenoceptor antagonist, completely inhibited the increase in $[Ca^{2+}]_i$ induced by 1 μM ISO, while the increase in $[Ca^{2+}]_i$ by 1 μM phenylephrine was not inhibited (Fig. 3A). In contrast, preincubation with 0.1 μM phentolamine, an α-adrenoceptor antagonist, did not inhibit the ISO-induced $[Ca^{2+}]_i$ response, and the increase in $[Ca^{2+}]_i$ by phenylephrine was prevented by the antagonist (Fig. 3B). Furthermore, when the antagonists were added during the sustained phase of the ISO-induced increase in $[Ca^{2+}]_i$, the increase was not affected by addition of 0.1 μM phentolamine, while subsequent addition of 0.1 μM propranolol decreased $[Ca^{2+}]_i$ to nearly the basal level (Fig. 3C).

To show that ISO caused an increase in $[Ca^{2+}]_i$ through activation of adenylate cyclase, the effect of forskolin, a direct activator of adenylate cyclase, on $[Ca^{2+}]_i$ was examined. As shown in Fig. 4, stimulation of ducts with 10 μM forskolin significantly increased $[Ca^{2+}]_i$, and the increase in $[Ca^{2+}]_i$ measured 10 s after stimulation was 52 ± 5 nM above the basal level (mean $\pm$ S.E.M., n = 6; $P<0.01$, compared with the basal). This change was comparable with that induced by 1 μM ISO. The cell-permeant cAMP analog DBcAMP (2 mM) also caused a small increase in the $[Ca^{2+}]_i$ of ductal cells, although the response was slower than that with ISO and forskolin (data not shown).

The cAMP-mediated cellular events are thought to be induced through activation of protein kinase A, and the effect of the protein kinase inhibitors, staurosporine and H-7, on the ISO-induced increase in $[Ca^{2+}]_i$ was examined. The ducts were preincubated for 5 min with 100 μM H-7 or 100 nM staurosporine and then stimulated with 1 μM ISO, but neither of these inhibitors affected the increase in $[Ca^{2+}]_i$ (data not shown). Furthermore, preincubation for 5 min with 10 μM KT5720, a more selective inhibitor for protein kinase A (12), did not also inhibit the ISO-induced increase in $[Ca^{2+}]_i$ (data not shown). The potent inhibitor for protein kinase A, H-89 (10 μM), itself caused a noticeable leakage of fura-2, and thus it was not possible to assess the effect of H-89 on the ISO-induced increase in $[Ca^{2+}]_i$.

To determine whether the ISO-induced increase in $[Ca^{2+}]_i$ can be attributed to a release of Ca^{2+} from intracellular Ca^{2+} stores, the changes in $[Ca^{2+}]_i$ were measured in a Ca^{2+}-free medium containing 0.2 mM EGTA. Stimulation of ducts with 1 μM ISO caused a transient increase in $[Ca^{2+}]_i$ that returned to the basal level (Fig. 5A), indicating that ISO induced a Ca^{2+} release from intracellular Ca^{2+} stores in ductal cells. Addition of 10 μM forskolin also caused a transient increase in $[Ca^{2+}]_i$ similar to that elicited by 1 μM ISO (Fig. 5A). No increase of $[Ca^{2+}]_i$ induced by forskolin was observed when the ducts had previously been stimulated with ISO (Fig. 5B), suggesting that forskolin mobilized Ca^{2+} from the ISO-sensitive Ca^{2+} store.

In salivary glands, the activation of α-adrenoceptors and muscarinic receptors induces Ca^{2+} mobilization from the inositol trisphosphate (IP_3)-sensitive Ca^{2+} store through phosphoinositide hydrolysis (1). To know whether ISO stimulates the IP_3-sensitive intracellular Ca^{2+} store, the

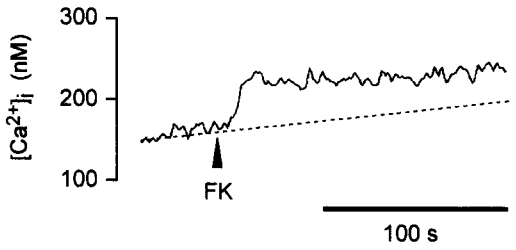

Fig. 4. Effect of forskolin (FK) on $[Ca^{2+}]_i$ in submandibular ducts. Ducts were stimulated with 10 μM FK in the presence of extracellular Ca^{2+}. The dotted line represents the apparent change in $[Ca^{2+}]_i$ in unstimulated ducts. The results are representative of 6 experiments.

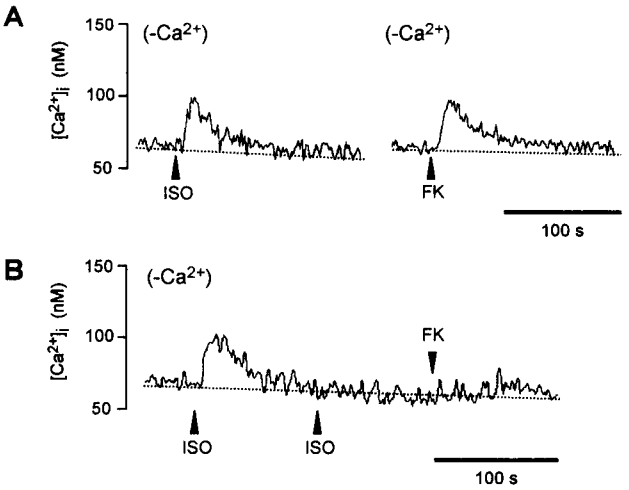

Fig. 5. Effects of ISO and FK on $[Ca^{2+}]_i$ in submandibular ducts in the absence of extracellular Ca^{2+}. A: Ducts were stimulated with 1 μM ISO or 10 μM FK in Ca^{2+}-free medium containing 0.2 mM EGTA. B: Ducts were stimulated with 1 μM ISO, followed by additional stimulation with 1 μM ISO. Then 10 μM FK was added at the time indicated by the arrowhead. The dotted lines represent apparent changes in $[Ca^{2+}]_i$ in unstimulated ducts. The results are representative of 6 experiments.

effects of sequential additions of carbachol or phenylephrine and ISO were examined in the absence of extracellular Ca^{2+}. When 1 μM ISO was added following a transient rise in $[Ca^{2+}]_i$ in response to 100 μM phenylephrine or 100 μM carbachol, there was no further increase in $[Ca^{2+}]_i$ (Fig. 6: A and B), suggesting that the ISO-sensitive Ca^{2+} store is also sensitive to phenylephrine and carbachol.

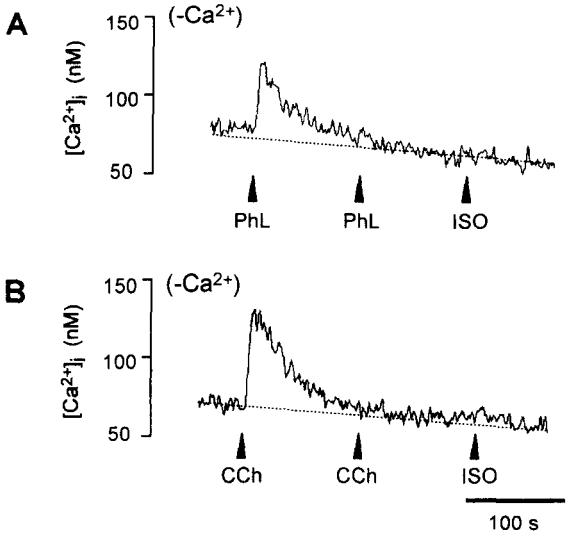

Fig. 6. Effects of sequential additions of PhL or carbachol (CCh) and ISO on $[Ca^{2+}]_i$ in submandibular ducts in the absence of extracellular Ca^{2+}. Ducts were stimulated with 100 μM PhL (A) or 100 μM CCh (B) in Ca^{2+}-free medium containing 0.2 mM EGTA, followed by additional stimulation with each agonist. Then 1 μM ISO was added at the times indicated by the arrowheads. The dotted lines represent changes in $[Ca^{2+}]_i$ in unstimulated ducts. The results are representative of 6 experiments.

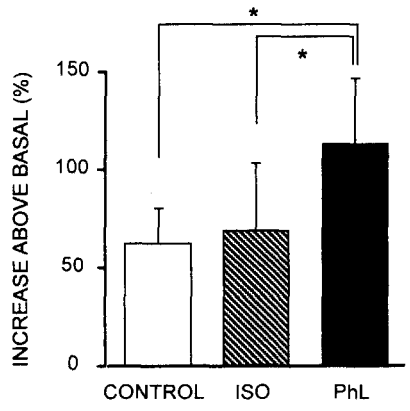

Fig. 7. Effect of ISO and PhL on formation of inositol trisphosphate (IP_3). [^{3}H]Inositol-labeled ducts were incubated for 10 min in the presence of 1 μM ISO or 1 μM PhL. In the control, the ducts were incubated without the agonists. Results are expressed as % increase above basal level (before incubation). Values are means ± S.E.M. of 8 experiments. *$P<0.05$, compared with the control and ISO-stimulated ducts.

To assess whether the ISO-induced Ca^{2+} release from intracellular stores is associated with phosphoinositide hydrolysis, the levels of IP_3 produced by 1 μM ISO and 1 μM phenylephrine were measured in the [^{3}H]inositol-labeled ductal cells (Fig. 7). In spite of the fact that 1 μM ISO can evoke a Ca^{2+} mobilization comparable with that induced by 1 μM phenylephrine (Fig. 3: A and B), incubation for 10 min with 1 μM ISO did not cause any detectable increase of cellular IP_3. In contrast, the level of IP_3 produced by 1 μM phenylephrine was significantly higher than the values in the control and ISO-stimulated ducts.

DISCUSSION

The β-adrenoceptor agonist ISO caused a small but significant increase in $[Ca^{2+}]_i$ in a preparation of purified submandibular ducts, while submandibular acini did not respond to ISO. We previously reported that the Ca^{2+} mobilization induced by high concentrations of ISO resulted from an activation of α-adrenoceptors rather than β-adrenoceptors (7). However, in the result obtained here, this is not the case, because the increase in $[Ca^{2+}]_i$ in submandibular ductal cells was induced by much lower concentrations (<1 μM) of ISO than the concentrations (approximately 100 μM) required for activating α-adrenoceptors (7, 8). In addition, the ISO-induced increase in $[Ca^{2+}]_i$ in the ductal cells was completely inhibited by the β-adrenoceptor antagonist propranolol but not by the α-adrenoceptor antagonist phentolamine. These results strongly support the idea that ISO is able to induce an increase in $[Ca^{2+}]_i$ through the activation of β-adrenoceptors in rat submandibular ducts.

Stimulation with ISO transiently increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, indicating that ISO mobilized Ca^{2+} from intracellular Ca^{2+} stores. As the Ca^{2+} mobilization is primarily mediated by IP_3, one explanation for the $[Ca^{2+}]_i$ response to ISO is that activation of β-adrenoceptors stimulated phosphoinositide hydrolysis resulting in the formation of IP_3. This hypothesis has been proposed by Xu et al. (13), based on the finding that pretreatment of submandibular ducts with the phospholipase C inhibitor U73122 prevented increases in $[Ca^{2+}]_i$ induced by ISO and forskolin. Xu et al. (13) suggest that forskolin may activate G proteins coupled to phospholipase C or may directly activate the enzyme to generate IP_3, although the effect of ISO on formation of inositol phosphates was not determined there. Our data on IP_3 formation show that stimulation with ISO does not cause a measurable increase of cellular IP_3 in rat submandibular ducts, which is consistent with the finding that ISO up to 10 μM had no effect on ductal IP_3 levels (5). These results do not support the hypothesis that the ISO-induced increase in

$[Ca^{2+}]_i$ observed in submandibular ducts is mediated through an activation of phosphoinositide hydrolysis.

The stimulation of salivary cells with ISO or forskolin results in an accumulation of cellular cAMP through activation of adenylate cyclase (1, 4), and it is reasonable to consider that the generated cAMP is involved in the ISO-induced Ca^{2+} mobilization in submandibular ducts. In addition, the cAMP analogue and the phosphodiesterase inhibitor also stimulate Ca^{2+} mobilization in rat submandibular ducts (5), leading to the idea that the Ca^{2+} mobilization is mediated by cAMP. Most effects of cAMP are generally believed to be associated with the activation of protein kinase A, but here the protein kinase inhibitors failed to inhibit the ISO-induced increase in $[Ca^{2+}]_i$. Dehaye et al. (5) have also reported that the inhibitors have no effect on the Ca^{2+} mobilization. These results suggest that the response of ductal $[Ca^{2+}]_i$ to ISO may not be mediated by the activation of protein kinase A, although it cannot be completely denied that treatment with the inhibitors was not sufficient to suppress the activation of protein kinase A.

In the present study, the stimulation with ISO failed to increase $[Ca^{2+}]_i$ after phenylephrine- or carbachol-induced depletion of intracellular Ca^{2+} stores (Fig. 6), suggesting that the increase in $[Ca^{2+}]_i$ mediated by cAMP is due to a Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores. In many cell types, the cAMP pathway has been demonstrated to modulate the agonist-induced Ca^{2+} responses (14–17). In rat parotid acinar cells, the cAMP pathway is able to enhance the Ca^{2+} release through a sensitization of IP_3 receptors (18, 19). Accordingly, one explanation for the ISO-induced Ca^{2+} mobilization is that the generated cAMP caused a Ca^{2+} release from the IP_3-sensitive Ca^{2+} stores by increasing the sensitivity of IP_3 receptors. It may be hypothesized that the sensitization of the IP_3 receptors would make it possible to respond to the resting level of cellular IP_3. However, there is no experimental support for this hypothesis.

Dehaye et al. (5) have described that ISO is able to cause increases in $[Ca^{2+}]_i$ even after the depletion of intracellular Ca^{2+} stores induced by carbachol, suggesting that the cAMP-sensitive intracellular Ca^{2+} stores are distinct from the IP_3-sensitive stores. This is contrary to the conclusion obtained by us and Xu et al. (13). Although there is no ready explanation for the discrepancy, it should be noted that the concentration (10 μM) of ISO was higher than that (1 μM) used in our and Xu et al.'s (13) studies. Therefore, further studies are necessary to determine the precise mechanism by which the accumulation of cAMP induces a Ca^{2+} release from intracellular Ca^{2+} stores.

The stimulus-response coupling in salivary ductal cells is only pooly understood compared with that in acinar cells, and the physiological significance of the Ca^{2+} mobilization elicited via β-adrenoceptors remains unclear. Kallikrein secretion from submandibular ducts is evoked mainly by α-adrenoceptor stimulation and only to a lesser extent by β-adrenoceptor stimulation (4). It may be possible that the increase in $[Ca^{2+}]_i$ evoked by activation of β-adrenoceptors is involved in limited secretion of kallikrein.

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