Characterization, localization and axial distribution of Ca^{2+} signalling receptors in the rat submandibular salivary gland ducts

Xin Xu, Julie Diaz, Hong Zhao and Shmuel Muallem*

Department of Physiology, University of Texas Southwestern Medical Centre, 5323 Harry Hines Boulevard, Dallas, TX 75235, USA

1. To characterize $[Ca^{2+}]_i$ signalling in salivary duct cells a procedure was developed for the rapid preparation and isolation of intralobular ducts, some of which had attached intercalated ducts. The isolated ducts retained agonist-induced Ca^{2+} signalling after permeabilization with streptolysin O (SLO).

2. The improved cell preparation technique was reflected in the repertoire and intensity of agonist responsiveness of the cells. Measurements of $[Ca^{2+}]_i$ in intact cells showed that all agonists previously reported to affect electrolyte transport by the submandibular salivary gland (adrenaline, carbachol, isoprenaline and forskolin) mobilized Ca^{2+} from internal stores and increased Ca^{2+} influx across the plasma membrane.

3. The use of the SLO-permeabilized ducts showed that all agonists, including isoprenaline and forskolin, mobilized Ca^{2+} exclusively from the inositol 1,4,5 trisphosphate (IP_3)-sensitive pool. However, in granular ducts only adrenaline mobilized the entire IP_3-sensitive pool whereas all other agonists mobilized only part of the pool.

4. All regions of the duct responded to substance P and the luminally secreted agonist ATP. Interestingly, the intercalated duct was most responsive to ATP and demonstrated only a minimal response to all other agonists. The granular region of the same duct and the extralobular duct always responded best to stimulation by adrenaline.

5. The perfused extralobular duct was used to show that adrenaline and carbachol stimulated the duct through the basolateral membrane whereas the receptors for ATP were localized in the luminal membrane of the duct. This suggests the presence of an ATP-dependent positive feedback loop in salivary duct with decreased activity along the ductal tree.

Fluid and electrolyte secretion by the submandibular salivary gland occurs in two steps (Thaysen, Thorn & Schwartz, 1954; Thaysen, 1960). First, a plasma-like fluid is secreted by the acinar cells and cells in the acinar intercalated duct region. Subsequently, the electrolyte composition of the primary fluid is modified by the granular, striated and extralobular ductal systems. The ducts reabsorb Na^+ and Cl^-, secrete K^+ and HCO^-_3 and reduce the osmolarity of the fluid (Young, Cook, van Lennep & Roberts, 1987). Salivary ion transport is under autonomic control. In the rat, both cholinergic and α-adrenergic stimulation inhibit Na^+ reabsorption and reduce the transepithelial potential (Young, Martin & Weber, 1970; Martin & Young, 1971; Martin, Fromter, Gebler, Knauf & Young, 1973; Schneyer, 1976, 1977; Denniss, Schneyer, Sucanthapree & Young, 1978). β-Adrenergic stimulation has variable effects depending on the agonist concentration (Martin & Young, 1971; Schneyer & Thavornthon, 1973; Dennis *et al.* 1978). Several gastrointestinal hormones, including substance P, also modulate ductal electrolyte transport in a manner similar to cholinergic stimulation (Denniss & Young, 1978; Young *et al.* 1987).

The mechanism and regulation of ductal electrolyte transport has been studied largely by determining the effect of ductal secretagogues on transepithelial potential and Na^+ reabsorption (Young *et al.* 1987; Case, Howorth & Padfield, 1988). However, in recent years techniques have been developed to examine these topics on the cellular level. Isolated ducts were used to show that isoprenaline stimulation increases cellular cAMP (Dehaye & Turner, 1991; Evans, Lau & Case, 1993). Measurement of $[Ca^{2+}]_i$ showed that cholinergic, α-adrenergic (Valdez & Turner

* To whom correspondence should be addressed.

1991; Dinudom, Porronnik, Allen, Young & Cook, 1993) and β-adrenergic (Dehaye, Valdez & Turner, 1993) stimulation, (but not substance P) caused an increase in $[Ca^{2+}]_i$. Cholinergic and α-adrenergic agonists were proposed to mobilize $[Ca^{2+}]_i$ from the IP_3-sensitive pool, whereas the effect of the β-adrenergic agonist isoprenaline on $[Ca^{2+}]_i$ was attributed to Ca^{2+} mobilization from an IP_3-independent pool (Dehaye *et al.* 1993).

In the present study we re-examined the signalling pathways of duct cells from the submandibular salivary gland in an effort to determine (a) the signalling pathways, (b) the axial distribution, and (c) the membrane localization of the receptors for the major ductal secretagogues. For the purpose of these studies we simplified and improved the technique of Dehaye & Turner (1991) for isolation of intralobular granular duct cells. The new digestion technique resulted in a significant number of granular ducts with attached intercalated ducts. The experimentally accessible perfused extralobular duct was used to provide direct membrane localization of the receptors for the major secretagogues. The intralobular ducts were used to develop an agonist-competent permeable cell system and show that all agonists, including isoprenaline, mobilize Ca^{2+} from the IP_3 pool. We identified previously unrecognized receptors for substance P and ATP in salivary duct cells. The localization, axial distribution and potency of response revealed the prominence of ATP receptors in the intercalated duct and of adrenaline receptors in all other regions of the submandibular ductal system.

METHODS

Materials and solutions

The acetoxymethyl ester form of fura-2 (fura-2 AM) and fluo-3 were purchased from Molecular Probes; adrenaline, isoprenaline, phentolamine, propranolol, ATP and carbachol were from Sigma; substance P was from Bachem (Torrance, CA, USA); Accudenz was from Accurate Chemical and Scientific Corporation (Westbury, NY, USA); collagenase P was from Boehringer Mannheim; streptolysin O was from Difco Laboratories (Detroit, MI, USA). The standard perfusion solution A contained (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 Hepes (pH 7·4, adjusted with NaOH).

Preparation of intralobular and intercalated ducts

A modified digestion procedure was used to obtain submandibular duct fragments consisting of intercalated and granular intralobular ducts. Sprague–Dawley male rats (100–150 g) were deeply anaesthetized by exposure to air saturated with ether and killed by cervical dislocation. The submandibular glands were removed to an ice-cold solution composed of solution A in which 10 mM sodium pyruvate replaced 10 mM NaCl and containing 0·02% soybean trypsin inhibitor and 0·1% bovine serum albumin (PSA). The glands were cleaned by injection of 5–10 ml PSA using a 25 gauge needle. After blotting, the capsule was removed and the gland finely minced. The minced tissue from two glands was transferred to 8 ml PSA containing 2·5 mg collagenase P (~1000 units mg^{-1}) and the flask was gassed with 100% O_2 and capped. After 2 min digestion at 37 °C with constant agitation at 200 cycles min^{-1} the tissue was partially dispersed by pipetting 5 times with a 5 ml plastic pipette tip. After gassing with O_2 the flask was capped and the digestion continued for an additional 8 min. During the last 4 min of digestion, the digest was vigorously shaken by hand every minute. The cells were washed twice with 30 ml PSA by 10 s centrifugation at 100 *g*, resuspended in about 6 ml PSA and kept on ice until use. When ducts from more than one rat were prepared, the minced tissue from each rat was digested in a separate flask. These cells (see Fig. 1*A* below) were used for microspectrofluorimetric measurement of $[Ca^{2+}]_i$ by photon counting or image acquisition and analysis.

Isolation of ducts and acini

Ducts and acini were separated on a one-step density gradient using the density matrix Accudenz. A density gradient was prepared by dissolving 1·15 g Accudenz in 4·6 ml PSA and 1 ml H_2O. This gradient had a density of 1·1 and an osmolarity of 310 mosmol kg^{-1}. The gradient was divided into two equal portions and placed in 15 ml plastic tubes. The digest from one gland, in 3 ml PSA, was layered on top of the gradient and the tubes were centrifuged for 6 min at 500 *g*. The acini were pelleted to the bottom of the tube, whereas the ducts remained in the interphase. The two cell types were collected, washed once and resuspended in PSA. The isolated ducts and acini were used to measured Ca^{2+} release after permeabilization with streptolysin O (SLO).

Microscopy

A suspension of submandibular gland cells or isolated acini and ducts in solution A were plated on polylysine-coated glass coverslips that formed the bottom of a perfusion chamber. After about 5 min incubation at room temperature (20–22 °C) the cells were perfused with 20 ml solution A to wash off the unattached cells and photographed.

Further analysis was done by fixation of the isolated cells and staining. The cells were packed by a 10 s centrifugation at 100 *g* and fixed by an overnight incubation in a solution containing 3% gluteraldehyde and 1% paraformaldehyde in a 100 mM cacodylate buffer (pH 7·4). The cells were then washed with buffer and dehydrated. Dehydration was achieved by succesive 30 min incubations in 50, 70, 90 and 100% alcohol and then 100% propylene oxide. Resin infiltration was then performed by incubation with various mixtures of propylene oxide–epon 812 as follows: 2:1 overnight, 1:1 for 4 h, 1:3 for 1 h, and full resin for 4 h. The sample was incubated overnight at 70 °C to obtain the resin block. For Toluidine Blue staining the blocks were sectioned at a 1 μm thickness on a Reichert ultramicrotome, and stained with 1% Toluidine Blue in 1% sodium borate. For electron microscopy 90 nm thin sections were made with the Reichter ultramicrotome and collected on an uncoated 100 mesh copper grids. The sections were contrasted by 10 min incubation with 3% uranylacetate in 50% ethanol, rinsed with H_2O, incubated for 5 min with lead citrate and finally rinsed with H_2O. The sections were viewed with a JEOL 1200 EM transmission electron microscope at 80 kV and images were photographed with a SO163 Kodak film.

Microdissection and perfusion of the extralobular submandibular duct

The procedure used to prepare the extralobular duct for perfusion was similar to that described before (Young *et al.* 1970; Denniss *et al.* 1978), while the perfusion set-up was similar to that used to perfuse the main pancreatic duct (Zhao, Star & Muallem, 1994) and study intracellular pH regulation in submandibular granular ducts (Zhao, Xin, Diaz & Muallem, 1995). In brief, a small segment (6–8 mm) of the duct was cannulated with a polyethylene tube of 100–120 μm i.d. and 220–240 μm o.d. The cannulated duct was cut and removed to a Petri dish containing PSA which was maintained at room temperature. A PSA solution containing 7·5 μM fura-2 AM was perfused into the duct lumen and the bath was washed twice with PSA to remove external dye. After 10–15 min incubation at room temperature, the duct lumen was flushed with 0·2 ml PSA. The cannulated duct was transferred to a perfusion chamber on the stage of an inverted microscope and the cannula connected to the lumen perfusion line. The lumen was perfused at a rate of 25 μl min^{-1}. The bath solution was passed through a heat exchanger to maintain the bath temperature at 37 °C and the bath was continuously perfused in the same direction as the lumen perfusate at a rate of 10–12 ml min^{-1}.

Fluorescence measurements

Intralobular and intercalated ducts and the acini were loaded with fura-2 by incubating the preparations for 20 min at room temperature with 2·5 μM fura-2 AM. The cells were washed once with PSA and kept on ice until use. All $[Ca^{2+}]_i$ measurements were carried out at 37 °C. Fura-2 fluorescence was recorded either by photon counting (perfused extralobular duct) or image acquisition and analysis using systems described previously (Loessberg-Stauffer, Zhao, Luby-Phelps, Moss, Star & Muallem, 1993; Zhao *et al.* 1994). In the case of the extralobular duct, single cells could not be clearly identified. Therefore, the fluorescence was recorded from an area equivalent to eight to twelve cells and as close as possible to the lumen-perfusing cannula. Fura-2 fluorescence was recorded at excitation wavelengths of 355 and 380 nm and the 355/380 ratios were calibrated to obtain $[Ca^{2+}]_i$ as detailed previously (Loessberg *et al.* 1993). In the case of image acquisition, images were recorded at a resolution of 4 s for each set of images and the fluorescence of single cells, cell clusters or different regions of a duct was analysed as desired.

Measurement of Ca^{2+} release in permeable cells

This was performed as described previously for pancreatic acini (Tortorici, Zhang, Xu & Muallem, 1994; Zhang *et al.* 1994). For these studies ducts from two rats were isolated by the Accudenz gradient. The ducts were suspended in 8 ml PSA and kept on ice. Before each Ca^{2+} release measurement, 1 ml of duct suspension was washed twice with a solution containing 140 mM KCl and 10 mM Hepes (pH 7·2, adjusted with NaOH) and once with the same solution treated with Chelex-100 (Sigma). The pellet was transferred to 500 μl of a warm peremabilization medium composed of the above Chelex-treated solution containing: 5 mM MgCl, 3 mM ATP, 10 mM creatine phosphate, 5 units ml^{-1} creatine kinase, 0·01 mM antimycin A, 0·01 mM oligomycin, 0·001 mM fluo-3, 0·02% soybean trypsin inhibitor and 3 mg ml^{-1} SLO. The permeabilization medium was in a thermostatically-controlled cuvette and the ducts were kept in suspension by continuous stirring. Fluo-3 fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. At the end of each experiment the signals were calibrated first by the addition of 1 mM Ca^{2+} to the cuvette to obtain maximum fluorescence (F_{max}) and then by the addition of 10 mM EGTA and 20 mM NaOH to obtain minimum fluorescence (F_{min}). The K_d was taken as 370 nM at 37 °C.

All experimental protocols to measure $[Ca^{2+}]_i$ or Ca^{2+} release from internal stores were performed at least 3 times with different cell preparations. When appropriate, data are given as means ± S.E.M. for the indicated number of experiments.

RESULTS

Light and electron microscopy

The various cellular preparations described in Methods were analysed by bright-field and electron microscopy. Figure 1*A* shows the mixture of ducts and acini obtained by the rapid digestion procedure. Most ducts contained granules, which appear as dark particles and distinguish the ducts as granular ducts. Striated ducts, which had the same general shape as the granular ducts, but lacked the dark granules, were observed occasionally in preparations from the submandibular and more often in preparations from the parotid gland. All experiments reported here are from granular ducts. Figure 1*B* and *C* shows a bright-field images of the isolated acini and ducts, respectively, obtained by the Accudenz gradient. Visual inspection and measurement of amylase and kallikrein content, markers of acini and ducts, respectively (Dehaye & Turner, 1991), showed less than 10% contamination of the duct preparation with acini and virtually 100% purified acini.

Close inspection showed that intercalated ducts remained attached to a significant number of intralobular ducts. The intercalated ducts were identified by their smaller size, presence at the tip of intralobular ducts (see several examples in Fig. 1*C*) and smooth appearance. In addition, small acinar fragments were sometimes attached to the tip of the structures, helping in their identification as intercalated interlobular ducts. Figure 2*A* shows two magnifications (inset, ×80; main panel, ×400) of an intralobular and intercalated duct. The smooth appearance and the size of the intercalated duct at the tip of the structure compared with the attached intralobular granular duct can be appreciated. To obtain better structural information on the different ducts, the cells were fixed and stained with Toluidine Blue. We have tried several fixation procedures and unfortunately fixation always resulted in fragmentation of the ducts and dissociation of most of the intercalated ducts. Three examples of granular ducts with attached intercalated ducts that remained intact are shown in Fig. 2*B*. The granules in the luminal side of the granular duct are clearly apparent. The intercalated duct cells are more packed and have no granules, accounting for their smooth appearance.

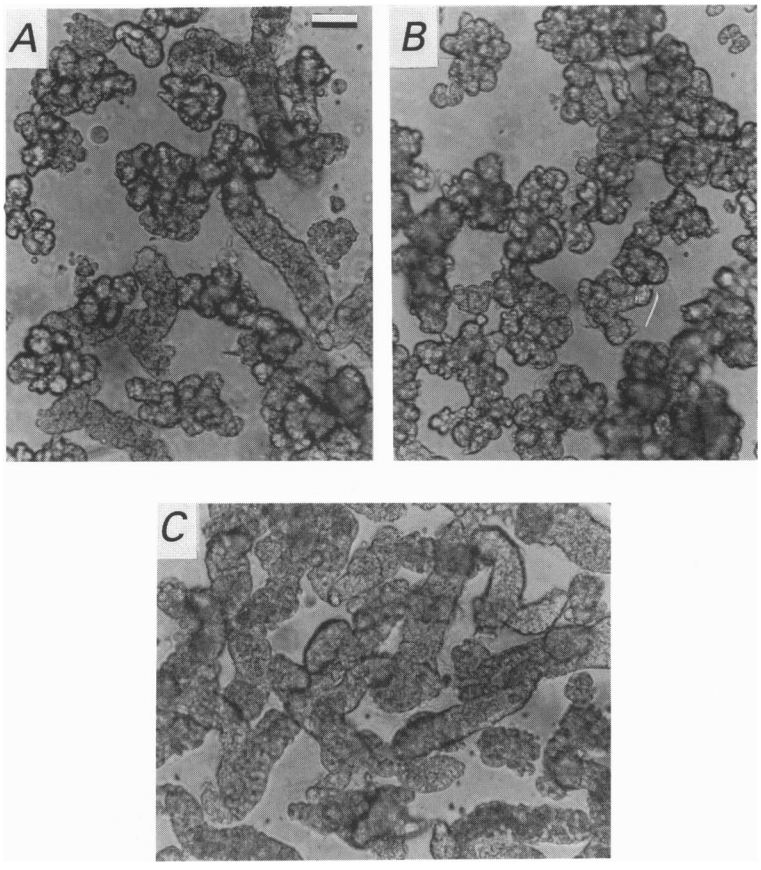

Figure 1. Light micrographs of isolated cells from the rat submandibular salivary gland
A shows the mixture of acini and ducts obtained after digestion of submandibular salivary glands. *B* and *C* show the acini and ducts, respectively, obtained by the density gradient. The scale bar indicates 125 μm and applies to all panels.

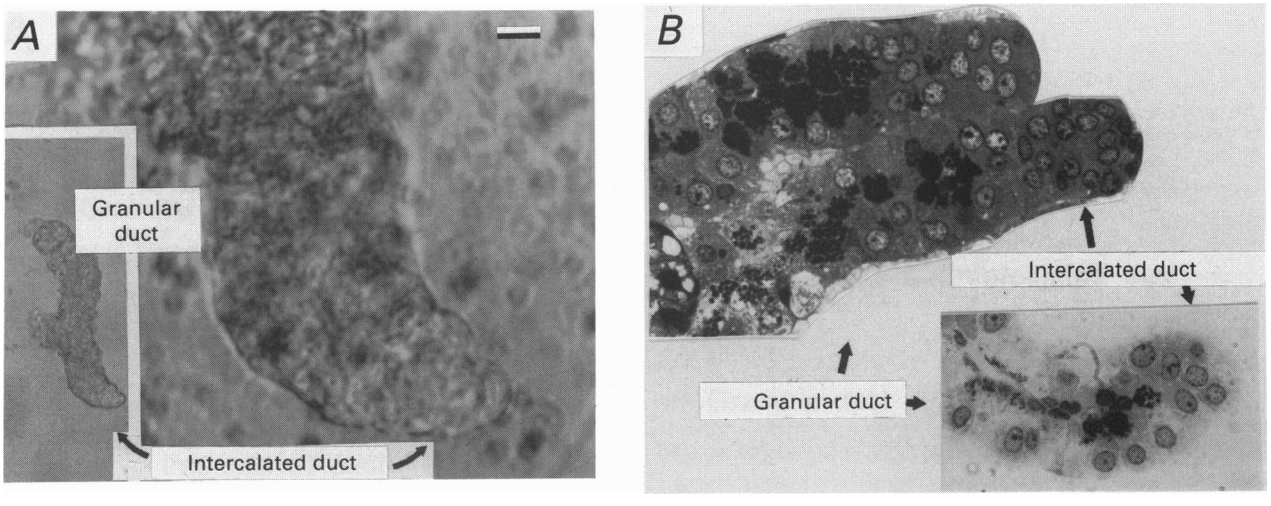

Figure 2. Light micrographs of live and fixed intralobular and intercalated ducts
The inset in *A* shows an intercalated duct attached to a granular intralobular duct (magnification ×80, as in Fig. 1). The main panel in *A* shows a × 400 magnification of the intercalated and intralobular region. Note the difference in appearance, size and granulation between the two ducts. Scale bar indicates 25 μm in both *A* and *B*. *B* shows fixed intercalated and intralobular ducts stained with Toluidine Blue. Note the presence of granules only in the granular duct cells and the packed structure of the intercalated duct.

The different structural features of the two ducts can also be seen in the electron micrographs shown in Fig. 3*A–C*. Again the intercalated duct is attached to a granular duct and the cells of this duct have no granules. In addition, the intercalated duct in Fig. 3*B* (and two others from the same block) showed a lower number of mitochondria compared with the granular duct cells attached to them. When $[Ca^{2+}]_i$ signalling was compared in the two ducts, a structure similar to that in Figs 2*A* and 3*A* was imaged and the fluorescence analysed from the tip (intercalated) and most adjacent, granule-containing (granular intralobular) regions.

Ca^{2+} signalling in intralobular ducts

The mixture of duct fragments and acini obtained after collagenase digestion (Fig. 1*A*) was used to characterize Ca^{2+} signalling of the new preparation of submandibular gland cells. The use of an image acquisition and analysis system allowed us to compare the response of acini and ducts from the same preparation. In agreement with previous reports, stimulation of granular intralobular ducts (Valdez & Turner, 1991; Dinudom *et al.* 1993) and acini (Dinudom *et al.* 1993) with adrenaline and carbachol increased $[Ca^{2+}]_i$ (Fig. 4*A–D*). However, ducts prepared by

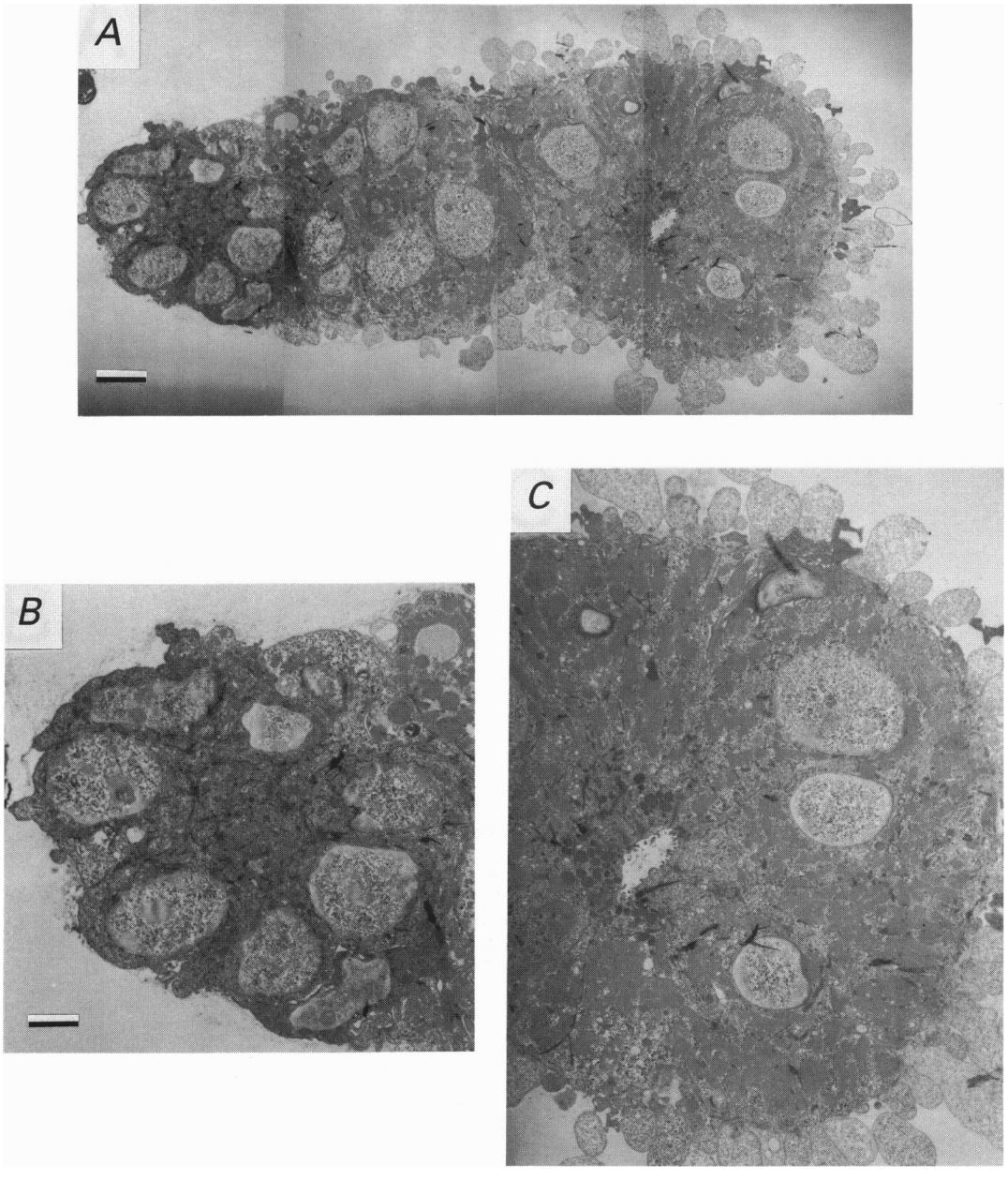

Figure 3. An electron micrograph of intralobular and intercalated ducts

A shows a ×2500 magnification of the duct (scale bar indicates 4 μm). *B* shows a ×5000 magnification of the intercalated region and *C* shows the same magnification of a granular region. Scale bar in *B* indicates 2 μm and also applies to *C*.

the simplified technique described in Methods were more responsive to agonists than those used in previous studies (Valdez & Turner, 1991; Dinudom *et al.* 1993). Thus, in our preparation, the EC_{50} values for the agonists were lower by about an order of magnitude and the maximal increase in $[Ca^{2+}]_i$ was about 6-fold higher than values for these parameters reported in cell suspensions (Valdez & Turner, 1991) or single cells (Dinudom *et al.* 1993). Figures 4*A* and *B* and 5*A* and *B* show that the ducts of the submandibular gland were more sensitive to adrenaline than the acini, whereas the acini were more sensitive to carbachol than the ducts. The EC_{50} values of the ducts and acini were 44 ± 6·7 and 786 ± 58 nM ($n = 6$), respectively, for adrenaline and 1·7 ± 0·3 and 0·42 ± 0·13 μM ($n = 6$), respectively, for carbachol. Maximal adrenaline stimulation increased ductal $[Ca^{2+}]_i$ by 886 ± 65 nM and acinar $[Ca^{2+}]_i$ by 324 ± 67 nM ($n = 6$). On the other hand, maximal carbachol stimulation increased submandibular ductal $[Ca^{2+}]_i$ by 352 ± 83 nM and acinar $[Ca^{2+}]_i$ by 946 ± 85 nM ($n = 6$). Figure 4*C* shows that isoprenaline increased $[Ca^{2+}]_i$ in duct but not acinar cells. The concentration-dependent curve for isoprenaline was monophasic with an EC_{50} of 4·8 ± 1·1 nM and a maximal increase in $[Ca^{2+}]_i$ of 213 ± 28 nM ($n = 4$) over resting levels (Fig. 5*C*). The EC_{50} for isoprenaline found in the present study is about three orders of magnitude lower than reported before (Dehaye *et al.* 1993).

An additional new finding of the present study is the response of the duct to ATP and substance P. Figure 4*D* shows that ATP increased $[Ca^{2+}]_i$ of the duct, but not the acini. In all experiments tested ($n = 16$) the granular intralobular ducts responded to 100 μM ATP with an increase in $[Ca^{2+}]_i$ to an average of 317 ± 12 nM ($n = 16$). The $[Ca^{2+}]_i$ response to 2–5 μM ATP was more variable and usually consisted of oscillations that lasted for the duration of stimulation without noticeable desensitization. A complete dose–response measurement similar to that in Fig. 4*D* was performed in eight experiments and resulted in an EC_{50} of 3·7 ± 0·58 μM for activation of the granular intralobular duct (Fig. 5*D*).

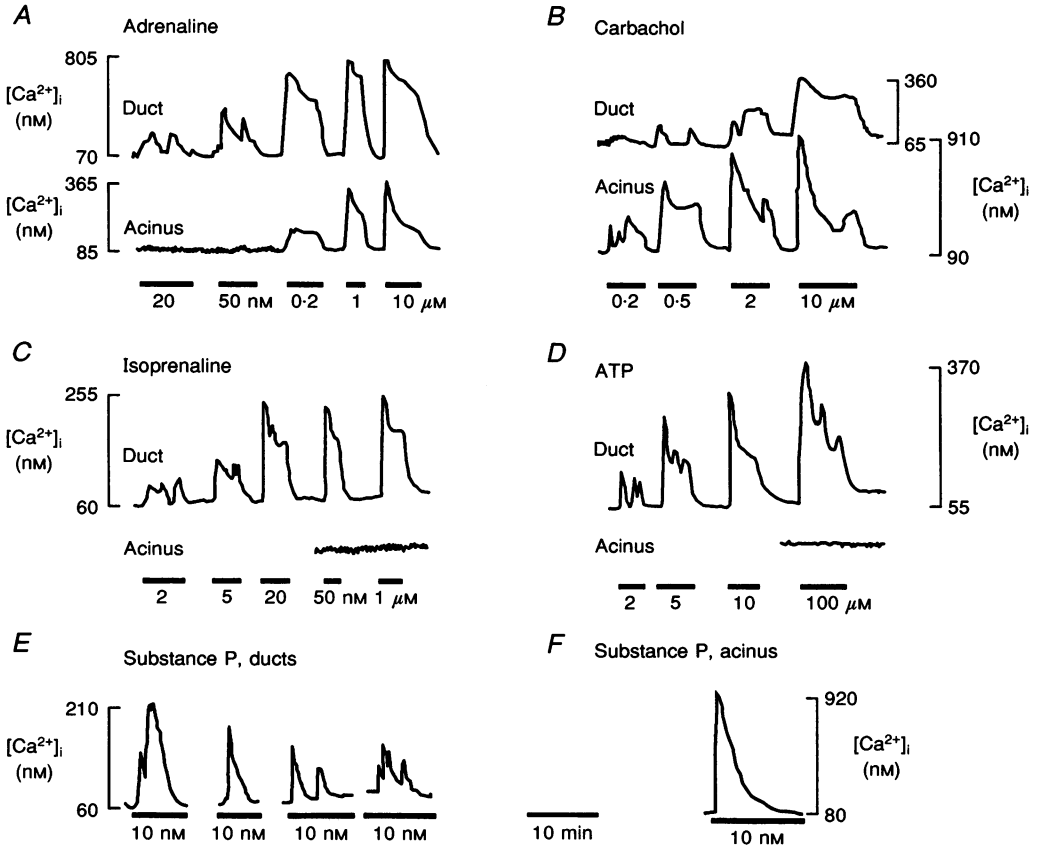

Figure 4. $[Ca^{2+}]_i$ signalling in intralobular ducts and acini

Fura-2-loaded ducts and acini in the same recording field were stimulated sequentially by the indicated concentrations of adrenaline (*A*), carbachol (*B*), isoprenaline (*C*), ATP (*D*) and substance P (*E* and *F*). The image ratios of ducts and acini were calibrated simultaneously by incubation with 5 μM ionomycin and 10 mM EGTA and then ionomycin and 10 mM Ca^{2+} to facilitate comparison between the two cell types. Note the different $[Ca^{2+}]_i$ scale for duct and acini in cells stimulated with adrenaline, carbachol and substance P.

Many (17/23 from 5 preparations) intralobular ducts responded to stimulation by substance P. However, the response of the duct was variable and significantly smaller than that of the acini in terms of the magnitude of the $[Ca^{2+}]_i$ increase (Fig. 4*E* and *F*). In addition, the response to substance P desensitized in that it was transient (Fig. 4*E*) and could not be elicited by a second substance P stimulation (of ducts or acini) 5–10 min after termination of the first stimulation (not shown). Similar desensitization to substance P was reported in guinea-pig pancreatic acini (Krim & Pandol, 1988). The variability and desensitization of the response precluded performing a dose–response curve on the same duct as for the other agonists.

To evaluate the receptor types involved in the response to adrenaline and isoprenaline we tested the effect of α- and β-blockers on the effect of these agonists. Figure 6*A* shows that exposure of an adrenaline-stimulated duct to 10 μM phentolamine reduced steady-state $[Ca^{2+}]_i$ from about 320 to 155 nM. Addition of the β-blocker propranolol in the presence of phentolamine was required to reduce $[Ca^{2+}]_i$ to resting levels. Accordingly, Fig. 6*B* shows that in the presence of phentolamine the response of the duct to adrenaline resembled that of isoprenaline. Figure 6*C* shows that propranolol completely inhibited the effect of isoprenaline and clearly reduced the steady-state $[Ca^{2+}]_i$ induced by adrenaline. Finally, Fig. 6*D* shows that 10 μM phentolamine had no effect on the response of duct cells to isoprenaline and phentolamine given with propranolol prevented the effect of adrenaline on ductal $[Ca^{2+}]_i$. Together these results demonstrate that isoprenaline activated the β-adrenergic receptors, whereas the response to adrenaline has α- and β-components. The α-adrenergic component dominated the effect of adrenaline on ductal $[Ca^{2+}]_i$.

To determine the pool from which isoprenaline, ATP and substance P mobilized Ca^{2+} we tested the effect of each

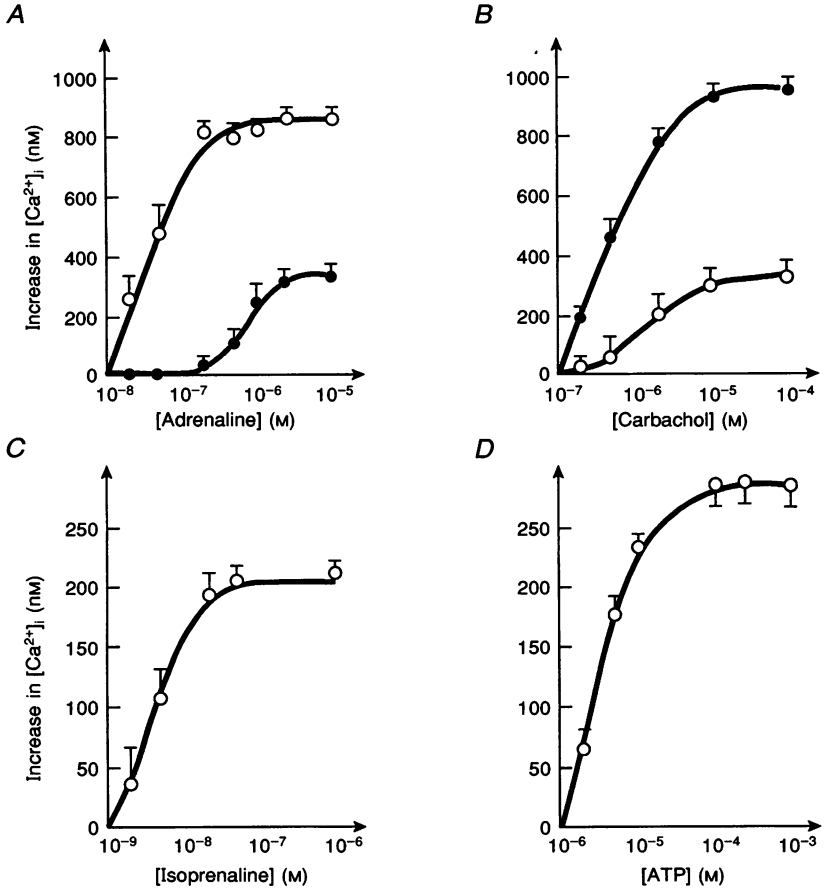

Figure 5. Concentration dependence of agonist-evoked $[Ca^{2+}]_i$ increase in intralobular ducts and acini

The experimental protocols were identical to those in Fig. 4. The figure shows the means ± S.D. of 6 (*A* and *B*), 4 (*C*) and 8 (*D*) separate experiments. ○, ducts; ●, acini. The EC_{50} for stimulation of duct cells by adrenaline was 44 ± 6·7 nM, for carbachol 1·7 ± 0·3 μM, for isoprenaline 4·8 ± 1·1 nM and for ATP 3·7 ± 0·58 μM. The EC_{50} for stimulation of acini by adrenaline was 786 ± 58 nM and by carbachol 0·42 ± 0·13 μM.

agonist before and after stimulation with adrenaline on $[Ca^{2+}]_i$. Figure 7 illustrates some of these findings. In general all agonists mobilized Ca^{2+} from internal stores (IS) and activated Ca^{2+} influx. The influx component dominated the increase in $[Ca^{2+}]_i$. Adrenaline was the most active agonist and largely depleted the IS of Ca^{2+}. Depletion of the agonist-sensitive pool with adrenaline prevented the effect of isoprenaline (Fig. 7*A*), carbachol, ATP and substance P (not shown) on $[Ca^{2+}]_i$. This was the case whether the cells were continuously stimulated with adrenaline (Fig. 7*A*) or if adrenaline was removed after stimulation of cells bathed in Ca^{2+}-free medium. In the absence of external Ca^{2+} isoprenaline transiently increased $[Ca^{2+}]_i$ and reduced the effect of subsequent stimulation with adrenaline by about $33 \pm 6\%$ ($n = 5$). Carbachol (Fig. 7*C*) and ATP (Fig. 7*D*) also mobilized Ca^{2+} from IS. However, these agonists were less effective than adrenaline in mobilizing IS Ca^{2+} and, accordingly, did not prevent but only reduced the effect of isoprenaline on $[Ca^{2+}]_i$.

Previous studies concluded that stimulation of submandibular salivary ducts with the β-adrenergic agonist mobilized Ca^{2+} from an IP_3-insensitive store, since prestimulation with carbachol did not prevent the effect of isoprenaline on $[Ca^{2+}]_i$, isoprenaline had minimal effect on cellular IP_3 levels and forskolin affected $[Ca^{2+}]_i$ similarly to isoprenaline (Dehaye *et al.* 1993). However, since in rat parotid acini isoprenaline and forskolin mobilize Ca^{2+} from the IP_3-sensitive pool (Horn, Baum & Ambudkar, 1988), we re-examined this suggestion first by testing the effect of the phospholipase C inhibitor U73122 on isoprenaline- and forskolin-induced $[Ca^{2+}]_i$ increase in submandibular ducts. Figure 8*A* and *B* shows that isoprenaline and forskolin mobilize the same intracellular Ca^{2+} pool, in that mobilization of the pool by one agent prevented the effect of stimulation with the second agent. Pretreatment of the ducts with 10 μM U73122 for 10 min prevented the effect of isoprenaline (Fig. 8*C*) and forskolin (Fig. 8*D*) on $[Ca^{2+}]_i$. The inhibition could be partially reversed by stimulation

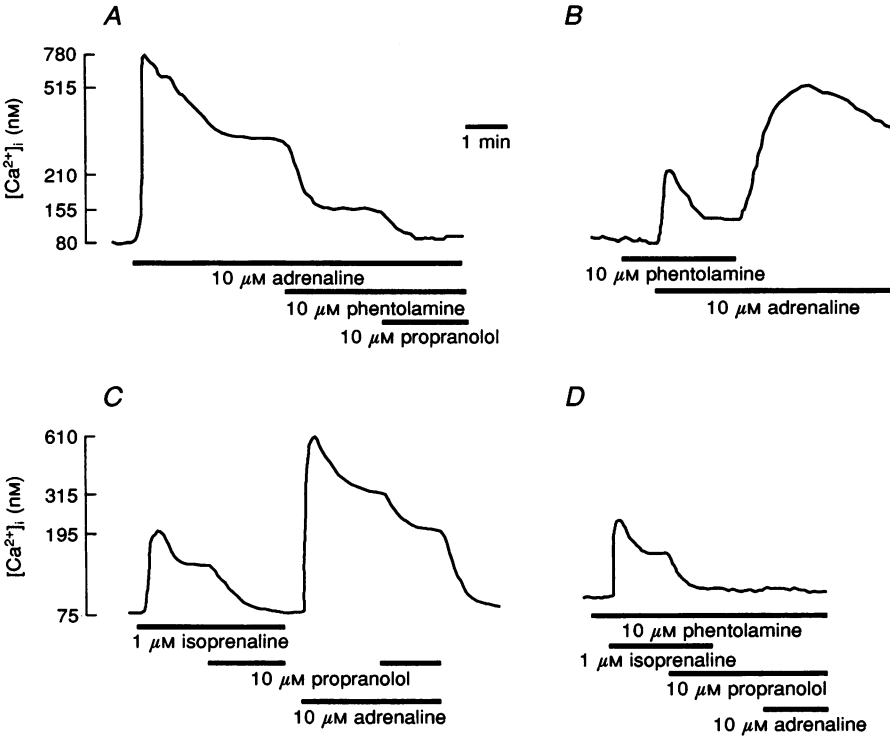

Figure 6. Effect of α- and β-blockers on the response of duct cells to adrenaline and isoprenaline

In the experiment shown in *A*, the duct was stimulated with 10 μM adrenaline. During continuous stimulation with adrenaline the duct was inhibited with 10 μM phentolamine and then phentolamine and 10 μM propranolol. In the experiment shown in *B*, the duct was incubated with phentolamine before stimulation with adrenaline. In the experiment shown in *C*, the duct stimulated with 1 μM isoprenaline was inhibited with 10 μM propanolol. After washing out the β-agonist and antagonist, the duct was stimulated with adrenaline and then exposed to 10 μM propanolol. In the experiment shown in *D* the duct was perfused with solutions containing the indicated concentrations of α- and β-agonist and blockers at the indicated times. Similar results to those in *A* and *C* were observed in five additional experiments, and to those in *B* and *D* in two additional experiments.

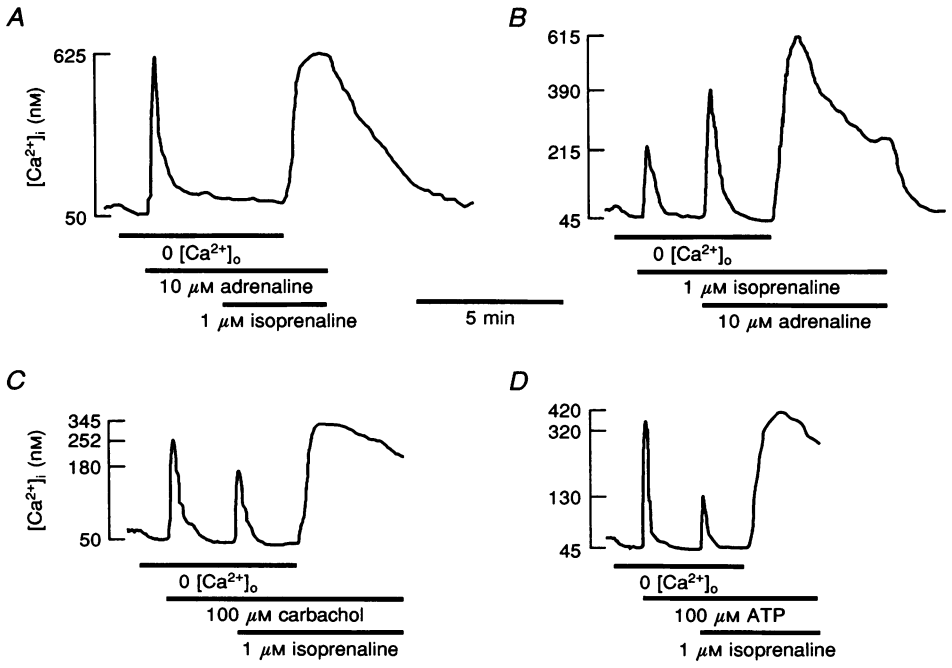

Figure 7. Effect of external $[Ca^{2+}]$ on agonist-mediated $[Ca^{2+}]_i$ increase in intralobular ducts

Fura-2-loaded ducts were perfused with Ca^{2+}-free solution A containing 0·1 mM EGTA before stimulation with the indicated concentration of the various agonists. The ducts were stimulated with adrenaline and then isoprenaline (*A*), isoprenaline and then adrenaline (*B*), carbachol and then isoprenaline (*C*) and ATP and then isoprenaline (*D*). In all experiments after stimulation with the second agonist, the effect of 1 mM external Ca^{2+} ($[Ca^{2+}]_o$) on $[Ca^{2+}]_i$ was tested by perfusing the stimulated cells with solution A.

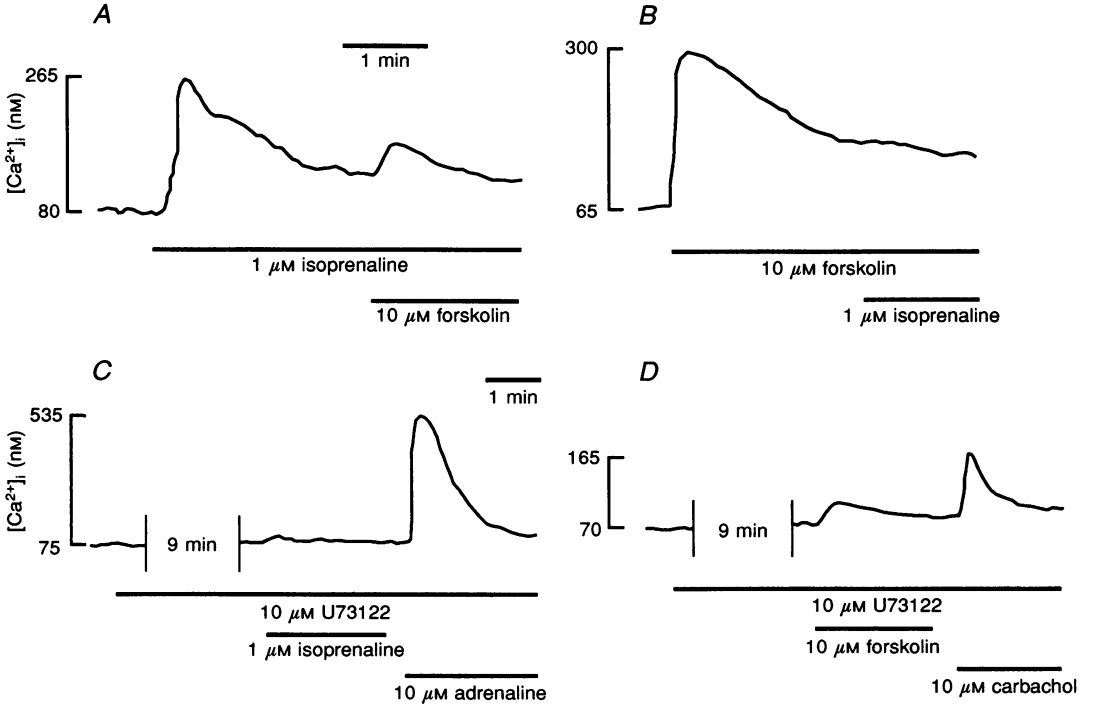

Figure 8. Effect of U73122 on Ca^{2+} signalling of duct cells

Ducts were stimulated by perfusion of solutions containing 1 μM isoprenaline with or without 10 μM forskolin (*A*), and 10 μM forskolin with or without 1 μM isoprenaline (*B*). Ducts were treated with 10 μM U73122 before stimulation with isoprenaline and adrenaline (*C*), or forskolin and carbachol (*D*). These experiments is representative of at least two others with similar results.

with agonists that cause a high increase in IP_3 levels (Dehaye *et al.* 1993). Thus, in U73122-treated cells adrenaline (Fig. 8*C*) and carbachol (Fig. 8*D*) were able to increase $[Ca^{2+}]_i$, although to significantly lower levels than those measured in the absence of U73122 (Figs 4 and 5).

Ca^{2+} release in permeable cells

To characterize further the Ca^{2+} pool mobilized by the various agonists we used permeabilized cells of streptolysin O (SLO) and measured whether exogenously added IP_3 and the agonists mobilize Ca^{2+} from the same or different pools and whether the effect of the various agonists can be inhibited by heparin. Figure 9 shows that ducts permeabilized with SLO retain the ability to respond to agonist stimulation. Addition of ducts to permeabilization medium resulted in Ca^{2+} uptake and reduction of medium Ca^{2+} from about 1500 to 163 ± 24 nM ($n = 46$). Stimulation of the ducts with any agonist in the absence of GTPγS caused minimal Ca^{2+} release from IS (Fig. 9*a* and *c*). Low concentrations of GTPγS alone caused minimal Ca^{2+} release. GTPγS added before (Fig. 9*b* and *d*), after (Fig. 9*a* and *c*) or together with the agonists (Fig. 9*e* and *f*) resulted in rapid and substantial Ca^{2+} release from IS. The requirement for GTPγS is likely to reflect the need to replace the GTP lost during permeabilization and activate the appropriate G proteins. As expected, adrenaline was more effective than isoprenaline in releasing Ca^{2+}. In

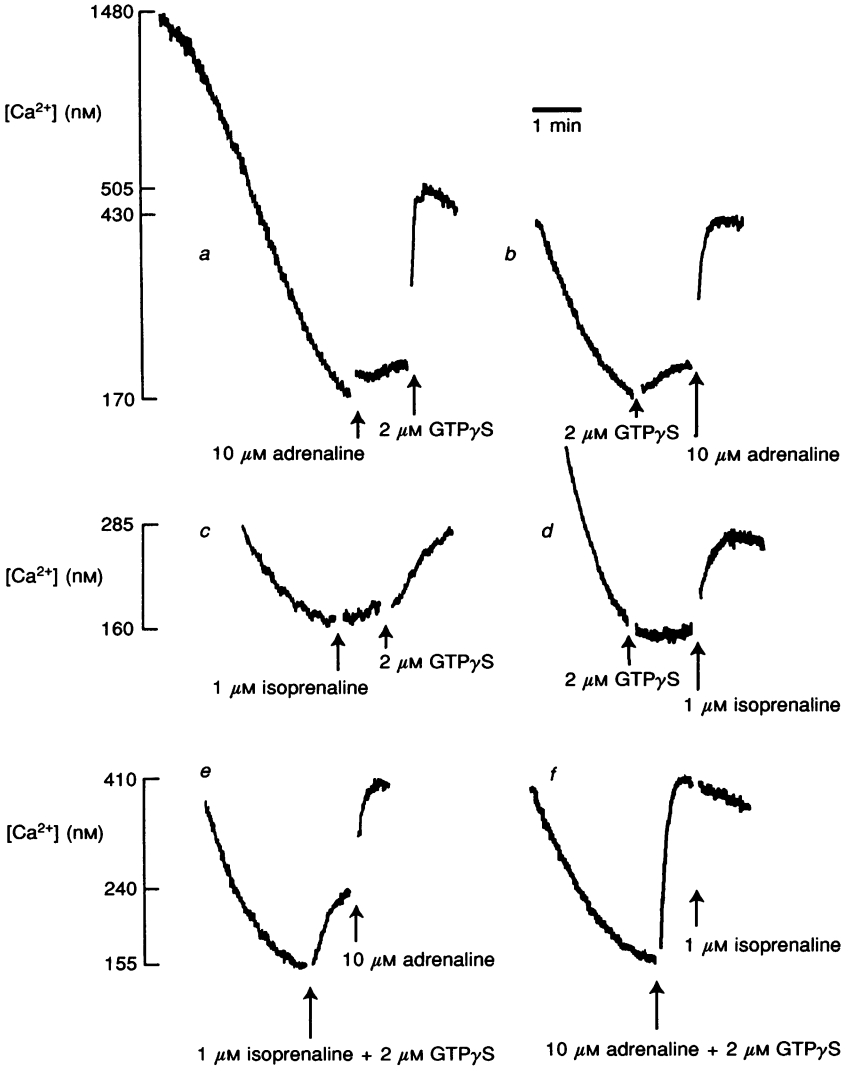

Figure 9. Agonist-mediated Ca^{2+} release from internal stores of SLO-permeabilized intralobular ducts

Isolated intralobular ducts (see Fig. 1*C*) were washed and added to permeabilization medium as detailed in Methods. After stabilization of medium $[Ca^{2+}]$ the ducts were stimulated with adrenaline (*a* and *b*) or isoprenaline (*c* and *d*) before (*a* and *c*) or after (*b* and *c*) addition of 2 μM GTPγS. The ducts were also stimulated with isoprenaline and GTPγS and then adrenaline (*c*) or adrenaline and GTPγS and then isoprenaline (*f*). Each experiment represents at least two others with similar results.

addition, stimulation of the cells with adrenaline prevented the effect of isoprenaline on Ca^{2+} release (Fig. 9*f*) and the effect of the two agonists was not additive (Fig. 9*e*), again suggesting that the two agonists mobilize Ca^{2+} from the same pool.

Figure 10 shows that the agonists and forskolin mobilize Ca^{2+} exclusively from the IP_3-sensitive pool. Thus, depletion of the pool with exogenous IP_3 prevented the effect of adrenaline (Fig. 10*a*), isoprenaline (Fig. 10*d*) and forskolin (not shown) on Ca^{2+} release. Prestimulation with adrenaline (Fig. 10*b*) or isoprenaline (Fig. 10*c*) reduced the effect of IP_3. The inhibitor of IP_3-mediated Ca^{2+} release heparin (Ghosh, Eis, Mullaney, Ebert & Gill, 1988; Zhao, Khademazad & Muallem, (1990), inhibited the effect of 2 μM IP_3 (not shown), adrenaline (Fig. 10*e*), isoprenaline (Fig. 10*f*) and forskolin (Fig. 10*h*). In fact, about 8 times less heparin was needed to inhibit the effect of isoprenaline than was needed to inhibit the effect of adrenaline (Fig. 10*e* and 10*f*). The protocols in Fig. 10 were used to show that carbachol and substance P also mobilized Ca^{2+} from the IP_3-sensitive pool of duct and acinar cells of the submandibular gland (not shown).

Ca^{2+} signalling in intercalated ducts

While characterizing Ca^{2+} responses in granular intralobular ducts we noticed that the intercalated ducts attached to the same granular ducts showed markedly different profiles of agonist-mediated signalling. Examples of these differences are illustrated in Fig. 11. Figure 11*A* shows that as low as 1 μM ATP induced clear $[Ca^{2+}]_i$ oscillations in intercalated ducts. At 2 μM ATP a marked increase in $[Ca^{2+}]_i$ to about 480 nM was observed. Stimulating the duct with 10 μM ATP increased $[Ca^{2+}]_i$ in the intercalated region to about 765 nM (Fig. 11*A*), and in the granular region to about 320 nM (not shown). In five experiments in which a complete dose–response procedure was performed the intercalated duct showed an EC_{50} for ATP of about 2·37 ± 0·31 μM and a maximal $[Ca^{2+}]_i$ increase at 100 μM ATP of 738 ± 66 nM. In the same ducts the granular region responded to ATP with an EC_{50} of about 3·3 ± 0·53 μM and maximal $[Ca^{2+}]_i$ increase of 356 ± 41 nM. Hence, the most significant difference in the response of the two regions of the duct to ATP was in the magnitude of Ca^{2+} mobilization rather than the affinity for the agonist.

The weak response of the intercalated region to two of the agonists tested is shown in Fig. 11*B* and *C*. Adrenaline at 10 μM (and up to 100 μM) caused a modest increase in $[Ca^{2+}]_i$ in the intercalated region and a strong increase in the granular region. That the modest response of the intercalated duct was not due to abnormal internal stores or signalling is shown by the vigorous response to a subsequent stimulation with ATP. Figure 11*C* shows that the response of the intercalated duct to carbachol was also reduced. Finally, in four out of four experiments from four different preparations, the intercalated region of the ducts did not respond to either isoprenaline (up to 10 μM) or substance P (up to 100 nM). The granular region of the same ducts responded to isoprenaline or substance P and the intercalated region responded to subsequent stimulation with ATP. Thus, it appears that ATP is the most prominent and dominant agonist in the intercalated duct.

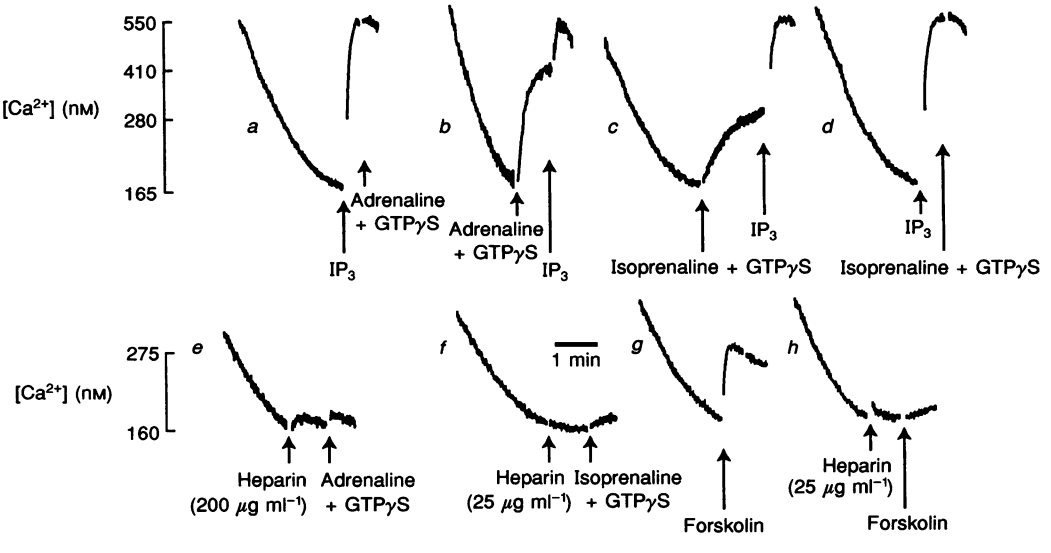

Figure 10. All agonists release Ca^{2+} from the IP_3-sensitive pool

Isolated ducts incubated in permeabilization medium were exposed to 2 μM IP_3 (*a–d*) before (*a* and *d*) or after (*b* and *c*) stimulation with the indicated agonist; 10 μM adrenaline; 1 μM isoprenaline and 2 μM GTPγS. The cells were treated with 200 (*e*) or 25 μg ml^{-1} (*f* and *h*) heparin before stimulation with 10 μM adrenaline (*e*), 1 μM isoprenaline (*f*) or 10 μM forskolin (*h*) and 2 μM GTPγS. The effect of 10 μM forskolin on Ca^{2+} release is also shown (*g*).

Localization of receptors

To determine from which side of the duct ATP triggers the $[Ca^{2+}]_i$ increase we cannulated and perfused the experimentally accessible extralobular duct of the submandibular gland. Loading fura-2 through the lumen ensured sufficient incorporation of the dye into the single layer of epithelial cells. Although single cells could not be visually identified through the connective tissue, a $[Ca^{2+}]_i$ dependent fluorescence change could be measured on exposure of the duct to high and low external Ca^{2+} and the Ca^{2+} ionophore ionomycin. The extralobular ducts were continuously perfused with separate bath and luminal solutions and agonists were included in the perfusate. Figure 12*A* shows that exposing the basolateral membrane of the duct to 100 μM ATP had no effect on $[Ca^{2+}]_i$. When applied to the luminal membrane the same concentration of ATP significantly increased $[Ca^{2+}]_i$. On the other hand adrenaline increased ductal $[Ca^{2+}]_i$ when applied to the basolateral side and had no effect when added to the luminal side (Fig. 12*A*). The response to carbachol showed the same sidedness as that of adrenaline (not shown). The relative response of the extralobular duct to adrenaline, carbachol and ATP was similar to that of the granular intralobular duct in that adrenaline caused the highest and ATP the lowest increase in $[Ca^{2+}]_i$.

The results in Fig. 12*B* suggest that although adrenaline- and ATP-dependent signalling reside in different membranes of duct cells, they stimulate the same cells to mobilize a common Ca^{2+} pool. Hence, stimulation of the duct with adrenaline through the basolateral membrane caused a marked increase in $[Ca^{2+}]_i$. When ATP was added to the lumen in the continuous presence of adrenaline in the bath, it had no further effect on $[Ca^{2+}]_i$ (Fig. 12*B*). The same duct responded to luminal ATP after removal of adrenaline from the bath and a Ca^{2+} reloading period of about 5 min.

The sidedness of the isoprenaline and substance P receptors could not be determined in the present studies since these agonists had no measurable effect on $[Ca^{2+}]_i$ of the extralobular duct when added to the bath and/or the lumen. However, since these agonists are known to affect electrolyte transport in the perfused extralobular duct (Young *et al.* 1987), it is likely that the lack of effect of

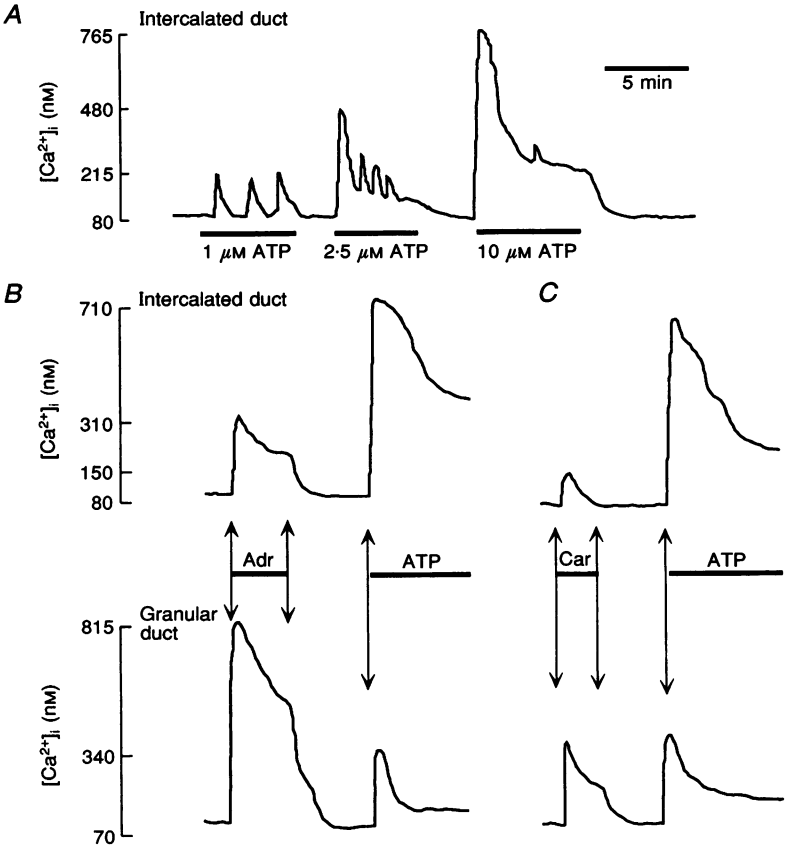

Figure 11. Comparison of Ca^{2+} signalling in intercalated and intralobular ducts

Ducts similar to that in Fig. 2*A* were stimulated by perfusion with solutions containing ATP at the concentrations indicated (*A*), 10 μM adrenaline and then 100 μM ATP (*B*) or 100 μM carbachol and then 100 μM ATP (*C*). The images from the intercalated and granular intralobular regions of the same ducts were then analysed and used to calculate $[Ca^{2+}]_i$.

these agonists on $[Ca^{2+}]_i$ was due to technical difficulties, including the time needed to prepare the duct for fura-2 recording and the time-dependent desensitization of substance P responses.

DISCUSSION

Ductal systems of various exocrine glands control the final electrolyte composition of the secreted fluid. The submandibular salivary gland has been extensively used as a model system to study the mechanism and regulation of electrolyte secretion by duct cells (Young *et al.* 1987). Most of the knowledge on these topics has been obtained by measurement of the effect of agonists on transepithelial potential and electrolyte transport in the perfused excretory duct. It has been difficult to obtain more direct information largely because of a lack of an adequate experimental system. However, recently a technique for the isolation of agonist-responding duct fragments from the submandibular salivary glands has been described (Dehaye & Turner, 1991) and used to characterize several aspects of signalling (Valdez & Turner, 1991; Dehaye *et al.* 1993) and ion transport (Paulais, Cragoe & Turner, 1994; Xu, Zhao, Diaz & Muallem, 1995) in granular and striated ducts. In the present studies we improved the duct isolation technique by changing the digestion procedure and gradient separation so that a digest was obtained within 15–20 min and isolated duct fragments within 35–40 min from the time of gland removal. Another advantage of our technique is that significant number of intercalated ducts remain attached to the granular ducts (Figs 1–3) so that their properties can be studied and compared with other regions of the ductal tree. A disadvantage of the present digestion procedure is that even when kept on ice, after about 3 h the acini and ducts started losing responsiveness to agonists and to fragment into smaller structures. Thus, for the present studies, cells from a digest were used within 3–4 h of their preparation. On the other hand, when the gradient-isolated ducts were washed and resuspended in DMEM/F12 medium containing 10% serum, they could be maintained in primary culture for at least 5 days without loss of signalling.

The freshly isolated ducts were particularly sensitive to stimulation by cholinergic α- and β-adrenergic agonists, with EC_{50} values 1 and 10 orders of magnitude,

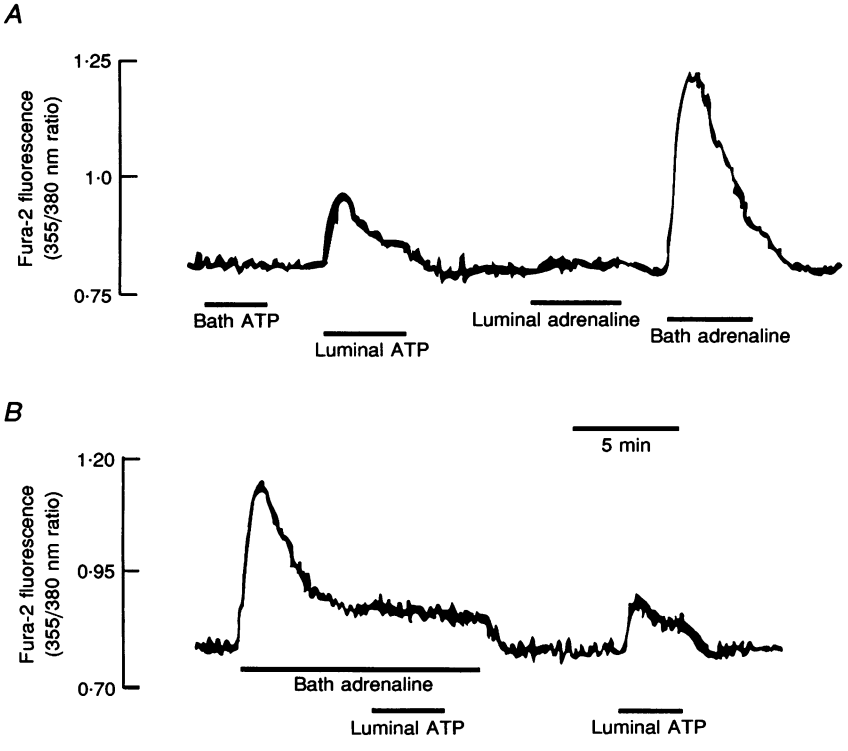

Figure 12. Localization of receptors in the perfused extralobular duct

Extralobular ducts were loaded with fura-2 and perfused with separate luminal and bath solutions. Where indicated in *A* the bath and then the luminal solutions contained 100 μM ATP. After removal of ATP the luminal and then basolateral sides were exposed to 10 μM adrenaline. In trace *B* the duct was stimulated with 10 μM adrenaline through the bath. Where indicated 100 μM ATP were added to the luminal solution, during and after stimulation with adrenaline. The experiment in *A* is representative of three others and in *B* of two others with similar results.

respectively, lower than reported previously (Valdez & Turner, 1991; Dehaye *et al.* 1993; Dinudom *et al.* 1993). Interestingly, the apparent affinity for isoprenaline in increasing $[Ca^{2+}]_i$ of rat ducts (4·8 nM, present studies) is about 400-fold lower than that reported for increasing cellular cAMP in rabbit ducts (2·1 μM; Evans *et al.* 1993). The effect of isoprenaline on the two second messengers and the large difference in the apparent affinities may account for the dual effect of this agonist on ductal electrolyte secretion (Young *et al.* 1987). At low concentrations isoprenaline stimulates, whereas at high concentrations it inhibits Na^+ and Cl^- reabsorption and K^+ and HCO^-_3 secretion (Schneyer & Thavornthon, 1973; Deniss, *et al.* 1978). This would suggest that an increase in $[Ca^{2+}]_i$ stimulates ductal secretion. However, many studies in the perfused isolated duct (listed in Young *et al.* 1987) showed that cholinergic and α-adrenergic stimulation inhibit Na^+ reabsorption. On the other hand, a more recent study by Case *et al.* (1988) in the rabbit mandibular gland showed that $[Ca^{2+}]_i$ is stimulatory and cAMP is inhibitory for ductal electrolyte transport. Our findings with isoprenaline signalling support this conclusion.

The isolated ducts were useful in identifying the intracellular pool from which the different agonists mobilized Ca^{2+}. Since isoprenaline and forskolin had no measurable effect on cellular IP_3 levels and they increased $[Ca^+]_i$ in duct cells stimulated with carbachol, it was concluded that isoprenaline and forskolin mobilize Ca^{2+} from an IP_3-insensitive pool (Dehaye *et al.* 1993). Three types of evidence show that this is not the case. First, in intact cells isoprenaline and adrenaline mobilize Ca^{2+} from the same intracellular pool. Isoprenaline mobilized internal Ca^{2+} in cells stimulated with carbachol since in granular duct cells carbachol released only about 40% of the Ca^{2+} present in the agonist-mobilizable pool. Second, the effect of isoprenaline and forskolin on $[Ca^{2+}]_i$ was inhibited by the phospholipase C inhibitor U73122, implying activation of this enzyme by the agonists to generate IP_3 and release Ca^{2+} from the IP_3-sensitive internal stores. A third and more direct evidence was obtained in SLO-permeable cells. Ducts (Figs 9 and 10) and acini (not shown) from the submandibular gland permeabilized with SLO retain intact Ca^{2+} signalling systems. Thus, in the presence of low concentration of GTPγS, which was not sufficient to mobilize Ca^{2+} by itself, the agonists caused Ca^{2+} release from internal stores. As expected from the experiments with intact cells, adrenaline was the most efficient agonist, whereas isoprenaline mobilized about 40% of the pool mobilized by adrenaline. Not only did discharge of the internal pool with IP_3 prevent the effect of all agonists tested (adrenaline, carbachol, substance P and isoprenaline), but heparin inhibited the effect of IP_3, the agonists and forskolin. Together the results provide strong evidence that all agonists, including isoprenaline, increase $[Ca^{2+}]_i$ by mobilization of Ca^{2+} stored in the IP_3-sensitive pool.

The signalling pathway of submandibular duct cells used by isoprenaline and forskolin to generate IP_3 is not known at present. However, the effect of U73122 indicates that both agonists activate phospholipase C. The β-adrenergic receptor is known to be coupled to the G_s and $G_{i/o}$ family of G proteins to regulate cellular cAMP (Gilman, 1987). Despite the effect of forskolin, this is unlikely to be the pathway used by isoprenaline to increase $[Ca^{2+}]_i$ since in SLO-permeable cells addition of up to 0·1 mM cAMP did not mobilize Ca^{2+} from the IP_3-sensitive pool (not shown). Recently it was shown that the β-adrenergic receptor can couple to two types of G proteins (Offermanns & Simon, 1995). Expression in COS-7 cells of different α-subunits from the G_s and G_q families, together with the β-adrenergic receptor, showed that the same receptor can couple to α_s to increase cellular cAMP and to α_{15} and α_{16} to increase IP_3 (Offermanns & Simon, 1995). It is therefore possible that in submandibular duct cells the β-adrenergic receptor is similarly coupled to two different G proteins. In this respect it is interesting that prestimulation of the ducts with forskolin prevented the effect of isoprenaline (Fig. 8), whereas prestimulation with carbachol and ATP, which increased ductal $[Ca^{2+}]_i$ more than forskolin (Figs 4 and 5), did not completely prevent the effect of isoprenaline (Fig. 7). This further suggests specific coupling of receptors to G proteins and compartmentalization of signalling complexes and pathways.

To increase $[Ca^{2+}]_i$, forskolin can activate G proteins coupled to phospholipase C or directly activate the enzyme to generate IP_3. The mechanism of action of forskolin is not known at present. However, it is of note that in permeable cells agonist-mediated Ca^{2+} release from the IP_3-sensitive pool required the addition of GTPγS to activate G proteins, whereas the effect of forskolin was independent of GTPγS (Figs 9 and 10). This would imply direct activation of phospholipase C by forskolin. However, much more work is needed to clarify this point.

A new finding made possible with the improved duct preparation technique was the response of duct cells to ATP and substance P. Two previous studies, using different techniques of duct isolation reported no effect of substance P on $[Ca^{2+}]_i$ (Dehaye & Turner, 1991; Evans *et al.* 1993). Although the substance P-evoked $[Ca^{2+}]_i$ signal in duct cells was smaller than that of acinar cells, it was consistently observed in intralobular ducts. However, the response was transient and rapidly desensitized. It is possible that such desensitization obscured the response of duct cells in previous studies. The effect of substance P found here may account for the effect of this agonist on electrolyte transport by the extralobular duct (Denniss & Young, 1978).

The response of duct cells to ATP is of particular interest. The ATP receptors were localized to the luminal side and showed heterogeneous axial distribution along the ductal tree. ATP was the major agonist acting on the intercalated

duct. Acinar cells secrete ATP to the lumen of this duct. Stimulation by luminal ATP can therefore serve as a local regulatory mechanism of intercalated duct cells by acinar cells. The granular intralobular and the extralobular ducts also responded to ATP. When stimulated, the granular duct is likely to secrete ATP stores in the peptic granules of this duct. In addition, ATP may be secreted to the lumen by cystic fibrosis transmembrane regulator channel or other luminal transport proteins containing ATP binding cassettes (for recent discussion see Al-Awqati, 1995). The luminal ATP would further stimulate the same duct cells by interacting with the purinergic luminal receptors. Thus, the response to luminal ATP can be considered a form of a positive feedback mechanism. In addition, ATP acting on the luminal membrane can provide a rapid and localized stimulation of proteins resident in this membrane. The most intriguing possibility is that ATP may be secreted by the granule-containing principal duct cells to act on the electrolyte transporting neighbouring intercalated cells. Such an effect can be used to co-ordinate the activity of the two cell types. Appropriate localization of transporters and transport activity to selective duct cells is needed to test this possibility.

In summary, in the present studies we developed techniques required to characterize Ca^{2+} signalling in salivary duct cells, show the extensive repertoire of agonists responsiveness of the ducts and the mechanism by which the agonists mobilize Ca^{2+} from the IP_3-sensitive pool. We also describe the axial distribution and membrane localization of receptors for several of the ductal secretagogues, which can account for several features of the regulation of ductal electrolyte transport by these agonists.

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Acknowledgements

We thank Daniel Mlcoch for technical assistance and Mary Vaughn for expert administrative support. This work was supported by NIH grants DK38938 and DK36591.

Received 20 April 1995; accepted 5 October 1995.