Characterization of Ca^{2+} Mobilization in the Human Submandibular Duct Cell Line A253 (44162C)

GUO H. ZHANG,[1] RONALD J. HELMKE, AND J. RICARDO MARTINEZ
Department of Pediatrics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Abstract. **The regulation of Ca^{2+} mobilization in the human submandibular duct cell line A253 was investigated by monitoring cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) using the Ca^{2+}-sensitive fluorescent indicator fura-2 and by measuring inositol 1,4,5-triphosphate (IP_3) formation. An increase in $[Ca^{2+}]_i$ was elicited by ATP, isoproterenol (IPR), or vasoactive intestinal polypeptide (VIP), but not by acetylcholine, norepinephrine, or substance P, suggesting that Ca^{2+} mobilization is regulated by P_2-purinergic, β_2-adrenergic, and VIP receptors. 1,4,5-IP_3 formation was significantly increased by ATP but not by the other agonists. Exposure of the cells to a membrane permeable cAMP analog, dibutyryl-cAMP, or to the adenylate cyclase activator forskolin induced a smaller increase in $[Ca^{2+}]_i$, indicating that the IPR-induced Ca^{2+} release is not mediated by cyclic AMP. Inhibition of the endoplasmic Ca^{2+}-ATPase with thapsigargin (TG) in Ca^{2+}-free medium induced a 207% increase in $[Ca^{2+}]_i$, and a subsequent exposure to ATP caused a further increase in $[Ca^{2+}]_i$ of 104%. Similarly, TG exposure after ATP induced a further Ca^{2+} release, suggesting that the TG-sensitive store and the IP_3-sensitive store do not overlap. Similar results were observed by sequential exposure to TG and IPR or to ATP and IPR. Ca^{2+} influx across the plasma membrane was enhanced after ATP or TG, but not after IPR. Our findings show a unique pattern of Ca^{2+} mobilization in the A253 cell line: (i) Ca^{2+} mobilization is regulated by P_2-purinergic, β_2-adrenergic, and VIP receptors; (ii) Ca^{2+} release is mediated by 1,4,5-IP_3 and probably by an unknown mediator; (iii) TG, P_2-, and β_2-agonists discharge separate Ca^{2+} stores; and (iv) ATP and TG, but not IPR, regulate Ca^{2+} influx.**

[P.S.E.B.M. 1997, Vol 216]

Ion transport in salivary ducts is regulated by the autonomic nervous system (1–4). Studies using the microperfusion technique have indicated that several neurotransmitters are involved in the regulation of ion transport in the duct system (3, 4). However, the intracellular signaling systems associated with electrolyte transport in salivary duct cells have not been clearly elucidated. Earlier studies indicate that NaCl reabsorption may be regulated by cyclic AMP (cAMP). Thus, activation of β-adrenergic receptors stimulates Na^+ reabsorption in isolated salivary ducts (5, 6), and this action is mediated by cAMP (6). In cultured salivary duct cells, β-adrenergic stimulation elicits an increase in cytosolic cAMP levels (7–9), and cAMP regulates Cl^- transport (7). It has also been demonstrated that Na^+ reabsorption in rat and rabbit submandibular ducts is inhibited by activation of muscarinic (10–12) or α_1-adrenergic receptors (13, 14). Since Ca^{2+} mobilization is induced by muscarinic or α_1-adrenergic stimulation in submandibular duct cells from the rat (15), mouse (16), and rabbit (6), it is likely that Ca^{2+} may be an intracellular messenger for the observed inhibition of NaCl reabsorption.

The submandibular duct cell line A253, which originated from a human squamous cell carcinoma (17), has been used to study the mechanisms of saliva modification by duct cells (18), to examine the biochemical and biological characteristics of chemotherapeutic agents (19–21), and to test the regulatory mechanisms of cancer cell growth and differentiation (22). However, the signal transduction systems of A253 cells have not been fully characterized, but it was

---

[1] To whom requests for reprints should be addressed at Department of Pediatrics, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284.

This work was supported by National Institutes of Health Grant DE09270.

Received February 26, 1997. [P.S.E.B.M. 1997, Vol 216]
Accepted June 1, 1997.

0037-9727/97/2161-0117$10.50/0

reported that this cell line possesses β_2-adrenergic receptors and lacks α-adrenergic and muscarinic-cholinergic receptors (8). Given the messenger role of Ca^{2+} in the modulation of salivary duct cell function and the somewhat unique receptor profile in A253 cells, the elucidation of how Ca^{2+} mobilization may be regulated in these cells can provide some clues about their signaling mechanisms. In this study, therefore, we investigated the regulation of Ca^{2+} mobilization in A253 cells by both agonists which increase IP_3 or cAMP formation. The results suggest that A253 cells have P_2-purinergic, β_2-adrenergic, and VIP receptors, and show IP_3- and an unknown mediator-sensitive Ca^{2+} release from separate stores.

Materials and Methods

Materials. Acetylcholine (ACh), ATP, bovine serum albumin (BSA, type V), N^6,2′-*O*-dibytyladenosine 3′,5′-cyclic monophosphate (db-cAMP), digitonin, ethylene glycol-*bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), forskolin, 3-isobutyl-1-methylxanthine (IBMX), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes), hyaluronidase (type V), ionomycin, isoproterenol (IPR), norepinephrine, substance P (Sub P), thapsigargin (TG), and vasoactive intestinal polypeptide (VIP) were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2/acetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR). All other chemicals used were of the highest grade available.

Solutions. Unless otherwise stated, all experiments used a modified Earle-Hanks' physiological salt solution (PSS) consisting of (in m*M*): 110 NaCl, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, 25 $NaHCO_3$, 20 Hepes, and 10 glucose. pH was adjusted to 7.4 after gassing with 95% O_2/5% CO_2 for at least 45 min. For the Ca^{2+}-free PSS, $CaCl_2$ was omitted.

Cell Culture. The A253 cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely grown on plastic tissue-culture T-75 flasks in McCoy's 5A medium (GIBCO, Grand Island, NY) containing 10% newborn bovine serum, 100 μg/ml penicillin and 100 U/ml streptomycin sulfate. The cells were cultured at 37°C in an atmosphere of 5% CO_2 in air. Medium was changed twice weekly. Subculture was conducted by discarding the medium, washing the cells with fresh serum-free medium, and exposing the cells to 0.25% trypsin and 1 m*M* EDTA for 5 min. Trypsinized cells were counted, centrifuged, resuspended in fresh medium and seeded to new flasks. For experiments, the confluent A253 cells were trypsinized as above and then rinsed twice with PSS. The cells were then resuspended in fresh PSS containing 1% newborn bovine serum and 2% trypsin inhibitors and incubated at 37°C for 2 hr before use.

Determination of Cytosolic Free Ca^{2+} Concentration. Cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-sensitive fluorescent indicator fura-2 as previously described (23, 24). Trypsinized A253 cells were loaded with fura-2 by incubation in 2 μ*M* fura-2/acetoxymethy ester for 20 min at 37°C. After loading, the cells were rinsed twice and kept at 37°C. A 2-ml aliquot of fura-2–loaded cells (1.5 × 10^6 cells/ml) was centrifuged at 50*g* for 2 min, resuspended in fresh medium containing 0.01% BSA, and placed in a 4-ml cuvette. $[Ca^{2+}]_i$ measurement was conducted using a PTI Deltascan fluorometer (PTI Inc., S. Brunswick, NJ). The excitation wavelengths used were 340 and 380 nm, and emission wavelength was 505 nm. Calibration of $[Ca^{2+}]_i$ was performed for each measurement trace by addition of 1 m*M* $CaCl_2$ and 50 μ*M* ionomycin to obtain the limiting ratio for the Ca^{2+} saturated form (R_{max}) of fura-2. Then, 0.0005% digitonin and 10 m*M* EGTA were separately added to obtain the limiting ratio for the unbound form (R_{min}) of fura-2. Fluorescence ratios of the 340/380 nm excitation and 505 nm emission were converted to $[Ca^{2+}]_i$ according to Grynkiewicz *et al.* (25) using 224 n*M* as K_d of fura-2 for Ca^{2+} at 37°C.

Ca^{2+} influx was measured in Ca^{2+}-free PSS. Cells were exposed to ATP, IPR, db-cAMP, thapsigargin, or vehicle for 5 min, and then 1 m*M* Ca^{2+} was then added. The linear portion of $[Ca^{2+}]_i$ changes after addition of Ca^{2+} was used to calculate Ca^{2+} influx rate (n*M*/min).

Determination of Inositol 1,4,5-Trisphosphate Production. Inositol 1,4,5-triphosphate (IP_3) formation was measured as previously described (24, 26, 27) using a radioligand assay kit (NEN, Boston, MA). Cells were treated with the indicated agonists or other agents. The reaction was initiated by addition of the agents and lasted for 30 sec. The reaction was terminated by adding an equal volume of 1 *M* ice-cold trichloroacetic acid (TCA). Samples were left on ice for 15 min, then centrifuged at 6000 rpm in a microfuge at 4°C for 5 min. The supernatants were mixed by vortexing with an equal volume of diethyl ether and the ether phase discarded. This step was repeated three times. Samples were then neutralized to pH 7.5 with 0.5 *M* $NaHCO_3$ prior to assaying IP_3 content. The level of IP_3 was measured according to the procedure recommended by NEN.

Data Presentation and Statistics. Results are presented as the mean ± SEM of separate determinations using different cell preparations. Comparisons were made using the unpaired Student's *t* test. *P* values <0.05 were considered significant.

Results

Effects of Agonists on $[Ca^{2+}]_i$. Exposure of A253 cells to 200 μ*M* ATP, a P_2-purinergic agonist, in the presence of extracellular Ca^{2+}, induced a 479% net initial increase in $[Ca^{2+}]_i$ (from 98 ± 11 n*M* to 567 ± 64 n*M*, $n = 5$) followed by a 68% sustained elevation (165 ± 16 n*M* at 5 min) (Table I and Fig. 1A). ATP triggered a 171% initial $[Ca^{2+}]_i$ increase in a Ca^{2+}-free PSS (from 73 ± 6 n*M* to 198 ± 21 n*M*, $n = 10$) (Table I and Fig. 1A). This increase was not due to activation of P_1-purinergic receptors since the P_1-agonist adenosine (2 m*M*) did not induce a significant

Table I. The Receptor Activation–Induced Changes in $[Ca^{2+}]_i$ in A253 Cells

$[Ca^{2+}]_o$	Agonist	*n*	$[Ca^{2+}]_i$ (n*M*) Basal	Initial	Sustained
1.2 m*M*	ATP (0.2 m*M*)	5	98 ± 11	567 ± 64[a]	165 ± 16[a]
	IPR (10 μ*M*)	6	84 ± 7	192 ± 22[a]	88 ± 7
	VIP (100 n*M*)	5	68 ± 8	101 ± 11[b]	73 ± 8
	ACh (10 μ*M*)	5	101 ± 13	105 ± 14	105 ± 15
	NE (10 μ*M*)	5	94 ± 11	112 ± 15	106 ± 15
	SP (100 n*M*)	5	107 ± 17	110 ± 16	112 ± 13
0 m*M*	ATP (0.2 m*M*)	10	73 ± 6	198 ± 21[a]	88 ± 8
	IPR (10 μ*M*)	9	78 ± 9	160 ± 13[a]	93 ± 9
	VIP (100 n*M*)	5	94 ± 9	127 ± 14	122 ± 14
	ACh (10 μ*M*)	6	83 ± 7	86 ± 6	78 ± 6
	NE (10 μ*M*)	5	100 ± 18	106 ± 19	119 ± 25
	SP (100 n*M*)	5	86 ± 11	89 ± 12	81 ± 7

[a,b] Compared with the basal level, $P < 0.05$ and 0.01, respectively.

increase in $[Ca^{2+}]_i$ (Fig. 1A). The ATP-induced $[Ca^{2+}]_i$ increase was concentration-dependent (Fig. 1B) with a $K_{1/2}$ of 42 μ*M* ATP.

Exposure to the β-adrenergic agonist isoproterenol (IPR, 10 μ*M*) in the presence of extracellular Ca^{2+} resulted in a 129% initial increase in $[Ca^{2+}]_i$ (from 84 ± 7 n*M* to 192 ± 22 n*M*, $n = 6$) (Table I and Fig. 2A). However, there was only a minimal (<5%) sustained elevation in $[Ca^{2+}]_i$ (Table I and Fig. 2A). In the absence of extracellular Ca^{2+}, IPR induced a 105% increase in $[Ca^{2+}]_i$ (from 78 ± 9 n*M* to 160 ± 13 n*M*, $n = 9$) (Table I and Fig. 2A). The IPR-elicited $[Ca^{2+}]_i$ response was completely abolished by the β-receptor antagonist propranolol (10 μ*M*, Fig. 2A), suggesting that the response is mediated by β-receptors. The IPR-elicited Ca^{2+} response was concentration-dependent with a $K_{1/2}$ of 3.3 n*M* IPR (Fig. 2B). Vasoactive intestinal polypeptide (VIP, 100 n*M*), another cyclic AMP-elevating neurotransmitter, elicited a 48% and 36% initial increase in $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+}, respectively (Table I).

Other agonists that have been reported to elicit $[Ca^{2+}]_i$ increase in salivary cells, such as the muscarinic agonist acetylcholine (ACh, 10 μ*M*), the α-adrenergic agonist norepinephrine (NE, 1 μ*M*), or the peptide substance P (SP, 100 n*M*), did not induce a change in $[Ca^{2+}]_i$ (Table I), suggesting that Ca^{2+} mobilization in A253 cells is not regulated by muscarinic, α-adrenergic, and substance P receptors.

Second Messengers Mediating Ca^{2+} Mobilization. IP_3 has been well recognized to be a major messenger mediating Ca^{2+} release from intracellular stores in most cell types, including salivary cells (28–30). To examine the role of IP_3 in the agonist-induced $[Ca^{2+}]_i$ increase, the effect of various stimuli on IP_3 formation was determined. As shown in Figure 3, A253 cells were stimulated with ATP, IPR, forskolin, or VIP. Only ATP significantly elevated cellular IP_3 levels (504% ± 77% of control). These results suggest that Ca^{2+} mobilization elicited by ATP is mediated by IP_3.

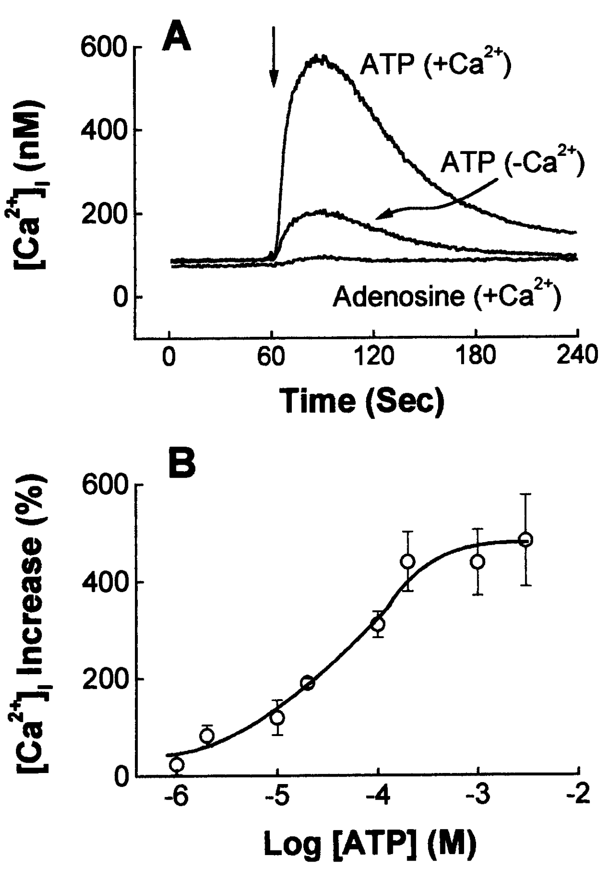

Figure 1. $[Ca^{2+}]_i$ changes in response to ATP or adenosine. (A) Fura-2–loaded A253 cells were suspended in Ca^{2+}-containing physiological salt solution ($+Ca^{2+}$) or a Ca^{2+}-free solution ($-Ca^{2+}$) and $[Ca^{2+}]_i$ was measured using a dual-wavelength fluorometer at 37°C. At the time indicated by the arrow 0.2 m*M* ATP (ATP) or 2 m*M* adenosine (adenosine) was added. Traces are representative of separate experiments using different cell preparations (ATP [$+Ca^{2+}$], $n = 5$; ATP [$-Ca^{2+}$], $n = 10$; adenosine [$+Ca^{2+}$], $n = 6$). (B) Concentration dependence of ATP-elicited $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured as described above in physiological salt solution. Values are the mean ± SEM of five separate experiments. The curve is produced by sigmoid curve (Boltzman) fit. The maximal $[Ca^{2+}]_i$ increase is 483% and $K_{1/2}$ is 42 μ*M* ATP.

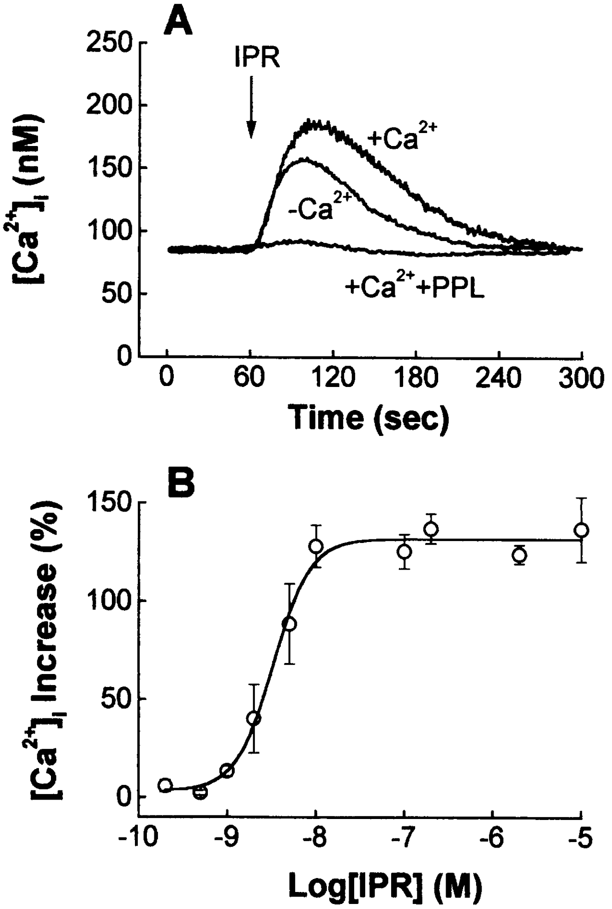

Figure 2. The isoproterenol (IPR)-induced $[Ca^{2+}]_i$ changes. (A) Fura-2–loaded A253 cells were stimulated with isoproterenol (IPR, 10 μM) at the time indicated by the arrow in Ca^{2+}-containing physiological salt solution ($+Ca^{2+}$), in a Ca^{2+}-free solution ($-Ca^{2+}$), or in Ca^{2+}-containing and 10 μM propanolol ($+Ca^{2+}$ + PPL). $[Ca^{2+}]_i$ was measured as described in Figure 1. Traces are representative of separate experiments using different cell preparations ($+Ca^{2+}$, $n = 6$; $-Ca^{2+}$, $n = 9$; $+Ca^{2+}$ + PPL, $n = 6$). (B) Concentration-dependence of IPR-stimulated $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in physiological salt solution as described above. Values are mean ± SEM of five separate experiments. The curve is produced by sigmoid curve (Boltzman) fit. The maximal $[Ca^{2+}]_i$ increase is 132% and $K_{1/2}$ is 3.3 nM IPR.

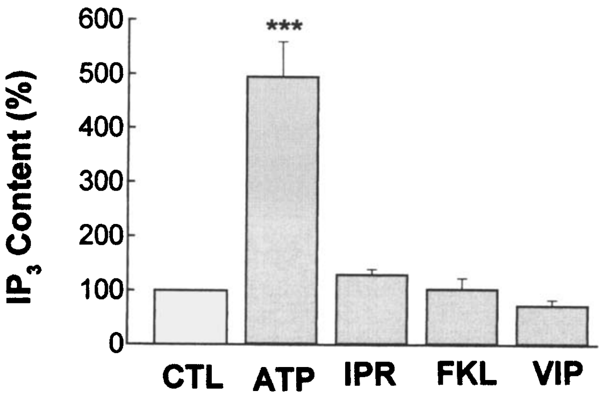

Figure 3. IP_3 formation induced by agonists or forskolin. A253 cells (2×10^6 cells/ml) were stimulated with 0.2 mM ATP (ATP), 10 μM isoproterenol (IPR), or 100 nM vasoactive intestinal peptide (VIP) for 30 sec, or with 5 μM forskolin (FKL) or dimethyl sulfoxide as vehicle control (CTL) for 1 min. After stimulation for the time indicated above, the reaction was terminated by addition of trichloroacetic acid and IP_3 content was measured as described in Materials and Methods. Values are the mean ± SEM of four separate determinations. ***Compared with control, $P < 0.005$.

Although the β-agonist IPR induced a significant increase in $[Ca^{2+}]_i$, it did not increase IP_3 formation. These results suggest that the IPR-elicited Ca^{2+} mobilization was mediated through a different signaling pathway. Since IPR is reported to induce a large (~250-fold) increase in cellular cyclic AMP level (8), the increase in $[Ca^{2+}]_i$ is likely to be mediated by cyclic AMP. Exposure of A253 cells to forskolin (5 μM), an activator of adenylate cyclase, in Ca^{2+}-free medium, induced a 14% initial increase in $[Ca^{2+}]_i$ (from 83 ± 10 nM to 95 ± 9 nM, $n = 5$) (Fig. 4A). In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), FKL (5 μM) induced a similar (14%) increase in $[Ca^{2+}]_i$ ($n = 5$, not shown). A membrane-permeable analog of cAMP, dibutyryl cyclic AMP (db-cAMP) was applied to test the role of cAMP in Ca^{2+} release. As shown in Figure 4B, 0.2 mM db-cAMP caused a 14% increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium (from 65 ± 3 nM to 74 ± 5 nM, $n = 7$). To corroborate this further, A253 cells were also exposed to 2 mM db-cAMP and 0.5 mM IBMX. This resulted in a 17% increase in $[Ca^{2+}]_i$ ($n = 5$, not shown). These results suggest that the cyclic AMP-associated signaling system is not the mediator for the large Ca^{2+} release induced by IPR in this submandibular duct cell line.

Ca^{2+} Release from Intracellular Stores. Ca^{2+} sequestration into the IP_3-sensitive intracellular Ca^{2+} store is Ca^{2+}-ATPase–dependent, and the Ca^{2+} release induced by thapsigargin (TG), an inhibitor of the endoplasmic Ca^{2+}-ATPase, appears to be from the same store as the IP_3-induced Ca^{2+} release in most cell types (31–33), including salivary acinar cells (26, 27, 34). To examine whether this is the case in this duct cell line, A253 cells were exposed to 3 μM TG, which induced a 352% increase in the peak $[Ca^{2+}]_i$ (from 85 ± 7 nM to 384 ± 36 nM; $n = 5$) (Fig. 5) in Ca^{2+}-containing PSS and a 207% increase in Ca^{2+}-free PSS (from 76 ± 7 nM to 219 ± 14 nM; $n = 14$) (Fig. 5). The latter result suggests the presence of a TG-sensitive Ca^{2+} store in this cell line. To examine further the Ca^{2+} storage sites that may be sensitive to ATP, TG, or IPR, the cells were sequentially exposed to ATP and TG, or IPR and TG, or ATP and IPR in a Ca^{2+}-free solution. As shown in Figure 6A, stimulation with ATP (200 μM) caused a 264.6% ± 30.5% increase in $[Ca^{2+}]_i$ ($n = 6$). The subsequent exposure to TG (3 μM) induced a 155.2% ± 17.8% further increase in $[Ca^{2+}]_i$ ($n = 6$) (Fig. 6A). A similar result was observed with these agents in the reverse sequence (Fig. 6B). Exposure of cells to 3 μM TG induced a 242.5% ± 41.1% increase in $[Ca^{2+}]_i$ ($n = 5$), and subsequent stimulation with ATP elicited a 103.5% ± 25.4% ($n = 5$) further increase in $[Ca^{2+}]_i$, suggesting a further release of Ca^{2+} (Fig. 6B). These results suggest that the Ca^{2+} release induced by ATP and TG is from separate stores that overlap partially.

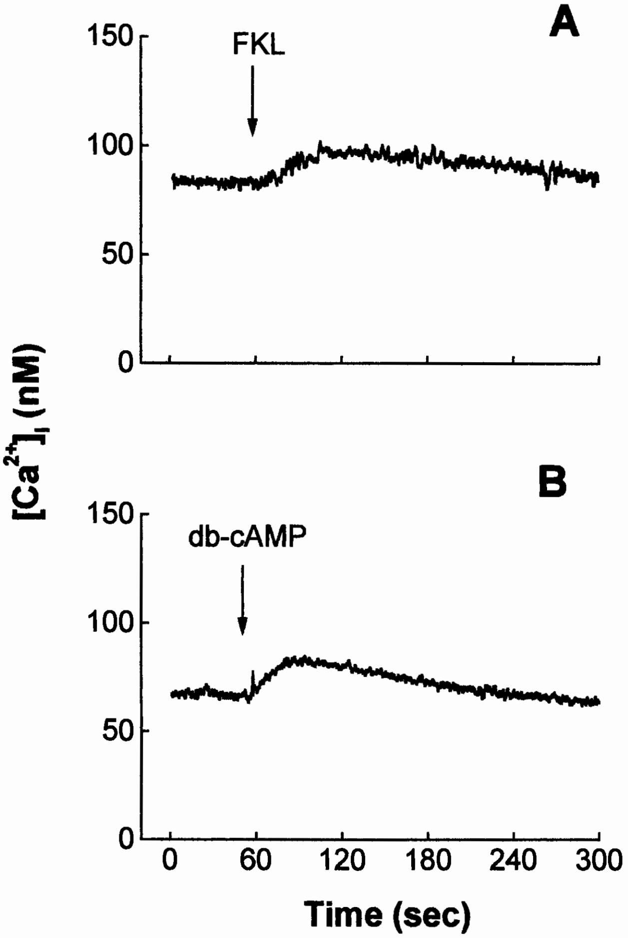

Figure 4. $[Ca^{2+}]_i$ changes induced by forskolin or dibutyryl-cAMP. Fura-2–loaded A253 cells were suspended in Ca^{2+}-free physiological salt solution and $[Ca^{2+}]_i$ was measured as described in Figure 1. At the time indicated by the arrow, 5 μ*M* forskolin (FKL) or 0.2 m*M* dibutyryl-cAMP (db-cAMP) was added. (A) $[Ca^{2+}]_i$ increase in response to forskolin. (B) $[Ca^{2+}]_i$ increase in response to dibutyryl-cAMP. Traces are representative of separate experiments (FKL, *n* = 5; db-cAMP, *n* = 7).

A similar Ca^{2+} release pattern was observed with IPR and TG. Stimulation first with IPR induced a 105% ± 16% increase in $[Ca^{2+}]_i$ ($n = 5$) (Fig. 6C), and a subsequent TG treatment caused a 230% ± 13% further increase in $[Ca^{2+}]_i$ ($n = 5$) (Fig. 6C). Exposure to TG first induced a 211% ± 25% increase in $[Ca^{2+}]_i$ ($n = 5$) (Fig. 6D). A subsequent stimulation with IPR (10 μ*M*) caused a 50% ± 10% further increase in $[Ca^{2+}]_i$ ($n = 5$) (Fig. 6D). These results suggest that the IPR- and TG-induced Ca^{2+} release is also from different stores.

Stimulation of A253 cells with ATP first triggered a 260% ± 25% increase in $[Ca^{2+}]_i$, and subsequent stimulation with IPR elicited a further 111% ± 28% increase ($n = 5$) (Fig. 6E). Stimulation of the cells in the reversed sequence (i.e., IPR first) resulted in a 93% ± 17% $[Ca^{2+}]_i$ increase ($n = 5$), and subsequent stimulation with ATP triggered a further 328% ± 37% increase ($n = 5$) (Fig. 6F). These results suggest that the Ca^{2+} release by IPR and by ATP was also from separate stores.

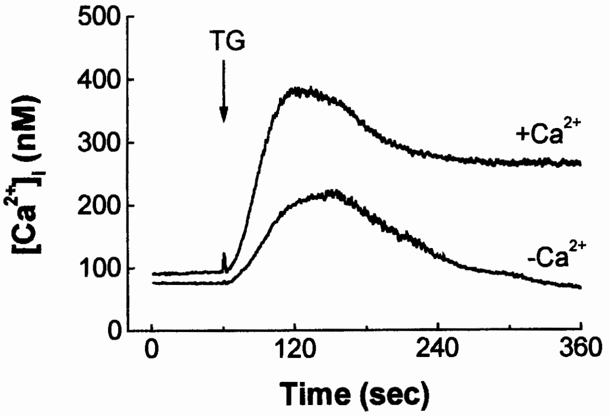

Figure 5. $[Ca^{2+}]_i$ changes induced by thapsigargin. Fura-2–loaded A253 cells were suspended in physiological salt solution (+Ca^{2+}) or Ca^{2+}-free PSS (−Ca^{2+}) and $[Ca^{2+}]_i$ was measured as described in Figure 1. At the time indicated by the arrow, 3 μ*M* thapsigargin (TG) was added. Traces are representative of separate experiments (+Ca^{2+}, *n* = 5; −Ca^{2+}, *n* = 14).

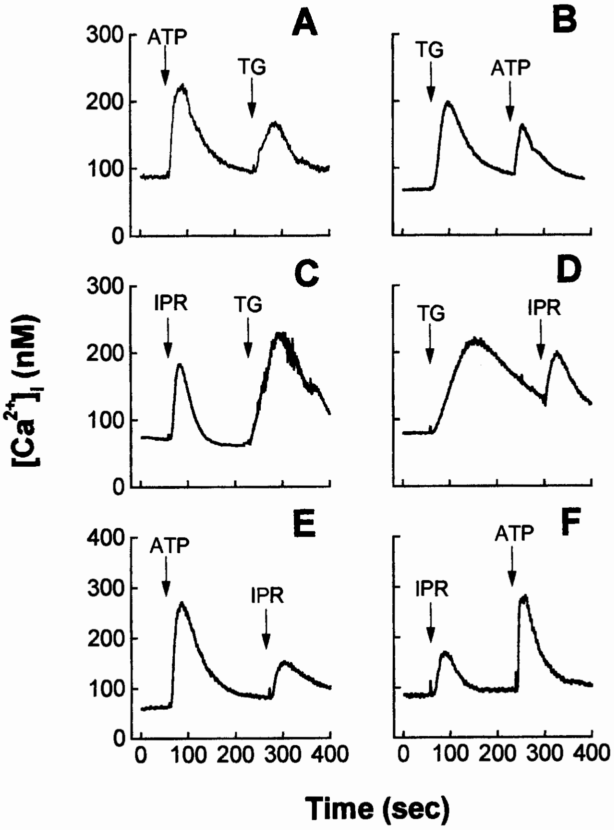

Figure 6. $[Ca^{2+}]_i$ changes induced by sequential exposure to ATP, thapsigargin, or isoproterenol. Fura-2–loaded A253 cells were suspended in Ca^{2+}-free physiological salt solution and $[Ca^{2+}]_i$ was measured as described in Figure 1. At the time indicated by arrows, 0.2 m*M* ATP (ATP), 3 μ*M* thapsigargin (TG), or 10 μ*M* isoproterenol (IPR) were added. (A and B) $[Ca^{2+}]_i$ changes induced by sequential exposure to ATP and TG (*n* = 6 [A]; *n* = 5 [B]). (C and D) $[Ca^{2+}]_i$ changes induced by sequential treatment with IPR and TG (*n* = 5 [C]; *n* = 5 [D]). (E and F) $[Ca^{2+}]_i$ changes induced by sequential stimulation with ATP and IPR (*n* = 5 [E]; *n* = 5 [F]).

Ca^{2+} Influx. $[Ca^{2+}]_i$ is regulated by both Ca^{2+} release from intracellular stores, which produces the main part of initial $[Ca^{2+}]_i$ increase, and Ca^{2+} influx across the plasma membrane, which maintains the sustained elevation (35–38). Therefore, we tested the effect of ATP, TG, IPR, and db-cAMP on Ca^{2+} influx. As shown in Figure 7, the initial rate of $[Ca^{2+}]_i$ increase in control (unstimulated) cells, after addition of 1 m*M* Ca^{2+} to cell suspensions that had been incubated in Ca^{2+}-free medium for 5 min, was 126 ± 18 n*M*/min ($n = 5$). The rate of $[Ca^{2+}]_i$ increase was significantly enhanced by ATP (287 ± 31 n*M*/min, $n = 5$; $P < 0.01$) and by TG (626 ± 82 n*M*/min, $n = 5$; $P < 0.001$), but not by IPR (97 ± 15 n*M*/min, $n = 5$) (Fig. 7) or by db-cAMP (164 ± 16 n*M*/min, $n = 5$; not shown).

Discussion

The results of this study show that the human submandibular duct cell line A253 has a unique receptor profile and a complex Ca^{2+}-mediated signal transduction system. Thus, Ca^{2+} mobilization is activated by ATP and by isoproterenol, but not by muscarinic, α_1-adrenergic, or substance P peptidergic agonists. This pattern of receptor-associated Ca^{2+} mobilization is different from that reported in rat salivary duct cells (Table II), which show Ca^{2+} mobilization in response to all three agonists (6, 15, 16, 39), and from that reported for other cultured salivary duct cell lines (Table II), which generally show responses to muscarinic and α-adrenergic stimuli (18). The significance of the unique receptor-associated Ca^{2+} responses in A253 cells is unclear but could be associated with a complex signaling system for the regulation of monovalent ion fluxes.

The Ca^{2+} mobilization induced by ATP in A253 cells is associated with P_2 purinoceptors (40), with IP_3 formation, with both Ca^{2+} release from internal stores and with Ca^{2+} influx across the plasma membrane. It is well established that extracellular ATP can activate P_2 purinoceptors in many cells (40), and that stimulation of P_{2u} and P_{2y} receptors leads to IP_3 formation and to release of Ca^{2+} from intracellular stores (40–43). Increases in $[Ca^{2+}]_i$ after exposure to ATP have been observed in rat submandibular acini (39, 44), in rat parotid cells (41, 45–47), and in the cultured submandibular duct cell line HSG-PA (42). P_2 purinoceptors thus operate in salivary cells of acinar and ductal origin through the IP_3/Ca^{2+} signal transduction pathway. Our results indicate that these receptors are also involved in IP_3-mediated Ca^{2+} mobilization in A253 cells.

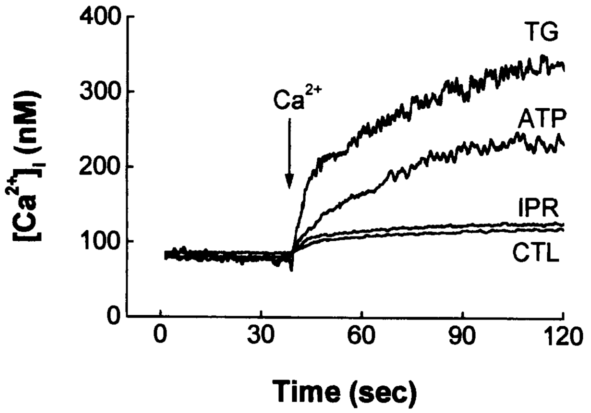

Figure 7. Effects of ATP, thapsigargin, or IPR on Ca^{2+} influx. Fura-2–loaded A253 cells were suspended in Ca^{2+}-free physiological salt solution, and $[Ca^{2+}]_i$ was monitored. The cells were exposed to 3 μ*M* thapsigargin (TG), 0.2 m*M* ATP (ATP), 10 μ*M* isoproterenol (IPR), or vehicle (CTL) for 5 min, then 1 m*M* Ca^{2+} was added at the time indicated by the arrow. Traces are representative of five separate experiments for each treatment after the addition of 1 m*M* Ca^{2+}.

Table II. $[Ca^{2+}]_i$ Response to Agonists in Different Cell Types

Cell types	$[Ca^{2+}]_i$ response					
	M	α_1	β	P_2	SP	VIP
Isolated duct cells	+	+	+	+	+	?
HSY	+	+	–	?	–	?
HSG	+	+	–	+	–	?
RSMT	+	+	–	?	–	?
A253	–	–	+	+	–	+

Note. +, positive response; –, negative response; ?, no data available.

An interesting observation in our study is that stimulation of β-adrenergic receptors also causes Ca^{2+} mobilization in A253 cells. The question whether β-agonists induce Ca^{2+} mobilization in salivary acinar cells is controversial (48–51), but effects of these agonists on IP_3 formation and Ca^{2+} release are thought to result from activation of α-adrenergic receptors by the high doses of drug used in some of these studies (51). By contrast, this type of agonist seems to induce Ca^{2+} release in salivary duct cells (39). Our results demonstrate that the Ca^{2+} response induced by isoproterenol in A253 cells is not due to activation of α-receptors since these cells lack these receptors (8) (Table I). Neither isoproterenol nor the other cAMP-generating agents had a significant effect on IP_3 formation (Fig. 3), suggesting that other mediators are involved. Although a Ca^{2+} release was induced by another cAMP-elevating neurotransmitter, VIP, the effect of forskolin and db-cAMP, which bypass the coupling of receptor and adenylate cyclase, was considerably smaller than that of isoproterenol. Furthermore, inhibition of cAMP breakdown by IBMX did not potentiate the Ca^{2+} response to db-cAMP or forskolin. These results suggest that cAMP is not the second messenger mediating the Ca^{2+} release induced by IPR, and that other intracellular signals may mediate the Ca^{2+} mobilization by β-agonists. It has been reported that cyclic ADP-ribose (cADPR) mediates Ca^{2+} release in many cell types including lacrimal acinar cells (52), which show a great similarity to salivary cells in functional responses. It is possible that the IPR-induced Ca^{2+} release in A253 cells is mediated by cADPR. Further investigations are required to address this issue. Another possibility is that GTP-binding proteins (G proteins) play a critical role in mediating Ca^{2+} release stimulated by IPR. G proteins have been shown to be involved in various signal transduction systems including regulatory signals of Ca^{2+} mobilization. In addition to the role in the activation of upstream elements such as phospholipases and adenylate cyclase, G proteins also participate directly in regulating some effectors. For example, the $\beta\gamma$ subunits of G protein can directly regulate Ca^{2+} channels in cardiac muscles and neurons (53, 54). Future studies will explore whether G proteins and cADPR play a role in the regulation of IPR-induced Ca^{2+} release in A253 cells.

Our results also suggest that A253 cells have multiple, complex Ca^{2+} stores. ATP induced IP_3 formation and Ca^{2+} release, suggesting that the Ca^{2+} store sensitive to ATP is discharged by IP_3. In many cells, the IP_3-sensitive store can also be emptied by the Ca^{2+}-pump inhibitor thapsigargin (33). However, in A253 cells, the sequential exposure to ATP and to TG resulted in two distinct and independent Ca^{2+} peaks (Fig. 6), which suggests that the release induced by each drug is from a different pool. The same was true when A253 cells were sequentially exposed to TG and IPR or to ATP and IPR (Fig. 6). These results suggest that these cells have at least three separate Ca^{2+} pools: (i) an IP_3-sensitive pool responsive to ATP; (ii) a TG-sensitive pool; (iii) an IPR-sensitive pool discharged by unknown mediators and insensitive to IP_3 or TG. The first two pools seem to be associated with Ca^{2+} entry probably of the capacitative entry type associated with emptying of the pool (37, 38) as both ATP and TG caused a significant Ca^{2+} influx. The separate, IPR-sensitive pool is not, however, associated with capacitative Ca^{2+} entry, as IPR failed to increase Ca^{2+} entry upon re-addition of Ca^{2+} (Fig. 7). The effects of this agonist are therefore on both a different Ca^{2+} pool and a different Ca^{2+} entry pathway than those responsive to ATP.

In summary, this study indicates that the human submandibular duct cell line A253 shows a unique model of Ca^{2+} regulation. (i) Ca^{2+} mobilization is primarily regulated by P_2-purinergic and β-adrenergic receptors, but not by muscarinic, α_1- or substance P receptors; (ii) three separate Ca^{2+} stores appear to be responsible for the Ca^{2+} release induced by ATP, IPR, and TG; and (iii) Ca^{2+} influx is activated by ATP stimulation, by the emptying of the TG-sensitive pool, but not by IPR stimulation. The physiological significance of this Ca^{2+} regulation model, specifically the relevance to electrolyte and water transport, a major function of salivary duct cells, requires further investigation.

We thank Stephanie Willis and Julie Wells for excellent technical assistance.

---

1. Anderson LC, Garret JR, Suleiman AH, Proctor GB, Chan K-M, Hartley R. In vivo secretory responses of submandibular glands in streptozotocin-diabetic rats to sympathetic and parasympathetic nerve stimulation. Cell Tissue Res **274**:559–566, 1993.
2. Garrett JR, Suleiman AM, Anderson LC, Proctor GB. Secretory responses in granular ducts and acini of submandibular glands in vivo to parasympathetic nerve stimulation in rats. Cell Tissue Res **264**:117–126, 1991.
3. Martinez JR. Ion transport and water movement. J Dent Res **66**:638–647, 1987.
4. Young JA, Van Lennep EW. Transport in salivary and salt glands. In: Giebisch G, Tosteson DC, Ussing HH, Eds. Membrane Transport in Biology. Berlin: Springer-Verlag, pp563–675, 1979.
5. Schneyer LH, Thavornthon T. Isoproterenol-induced stimulation of sodium absorption in perfused salivary duct. Am J Physiol **224**:136–139, 1973.
6. Evans RL, Lau KR, Case RM. Structural and functional characterization of striated ducts isolated from the rabbit mandibular salivary gland. Exp Physiol **78**:49–64, 1993.
7. He XJ, Ship J, Wu XZ, Brown AM, Wellner RB. β-Adrenergic control of cell volume and chloride transport in an established rat submandibular cell line. J Cell Physiol **138**:527–535, 1989.
8. Marmary Y, He X, Hand AR, Ship JA, Wellner RB. β-Adrenergic responsiveness in a human submandibular tumor cell line (A253). In Vitro **25**:951–958, 1989.
9. Patton LL, Pollack S, Wellner RB. Responsiveness of a human parotid epithelial cell line (HSY) to autonomic stimulation: Muscarinic control of K^+ transport. In Vitro **27A**:779–785, 1991.
10. Denniss AR, Young JA. The action of neurotransmitter hormones and analogues and cyclic nucleotide and theophylline on electrolyte transport by the excretory duct of the rabbit mandibular gland. Pflugers Arch **357**:77–89, 1975.
11. Field MJ, Young JA. Kinetics of Na transport in the rat submaxillary main duct perfused in vitro. Pflugers Arch **345**:207–220, 1973.
12. Martin CJ, Frömter E, Gebler E, Knauf B, Young JA. The effects of carbachol on water and electrolyte fluxes and transepithelial electrical potential differences of the rabbit submaxillary main duct perfused in vitro. Pflugers Arch **341**:131–142, 1973.
13. Denniss AR, Schneyer LH, Sucanthapree C, Young JA. Action of adrenergic agonists on isolated excretory ducts of submandibular glands. Am J Physiol **235**:F548–F556, 1978.
14. Schneyer LH. Sympathetic control of Na,K transport in perfused submaxillary main duct of rat. Am J Physiol **230**:341–345, 1976.
15. Valdez IH, Turner RJ. Effects of secretagogues on cytosolic Ca^{2+} levels in rat submandibular granular ducts and acini. Am J Physiol **261**:G359–G363, 1991.
16. Dinudom A, Poronnik P, Allen DG, Young JA, Cook DI. Control of intracellular Ca^{2+} by adrenergic and muscarinic agonist in mouse mandibular ducts and endpieces. Cell Calcium **14**:631–638, 1993.
17. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Parks WP. In vitro cultivation of human tumors: Establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst **51**:1417–1423, 1973.
18. Patton LL, Wellner RB. Established salivary cell lines. In: Dobrosielski-Vergona K, Ed. Biology of the Salivary Glands. Boca Raton, FL: CRC Press, pp319–341, 1993.
19. Reiss M, Stash EB, Vellucci VF, Zhou Z-L. Activation of the autocrine transforming growth factor a pathway in human squamous carcinoma cells. Cancer Res **51**:6254–6262, 1991.
20. McGuire JJ, Bergoltz VV, Heitzman KJ, Haile WH, Russell CA, Bolannowska WE, Kotake Y, Haneda T, Nomura H. Novel 6,5-fused ring heterocyclic antifolates: Biochemical and biological characterization. Cancer Res **54**:2673–2679, 1994.
21. Lazo JS, Braun ID, Meandzija B, Kennedy KA, Pham ET, Smaldone LF. Lidocaine potentiation of bleomycin A_2 cytotoxicity and DNA stran breakage in L1210 and human A-253 cells. Cancer Res **45**:2103–2109, 1985.
22. Lazo JS, Braun ID, Labaree DC, Schisselbauer JC, Meandzija B, Newman RA, Kennedy KA. Characterization of bleomycin-resistant phenotypes of human cell sublines and circumvention of bleomycin resistance by lilomycin. Cancer Res **49**:185–190, 1985.
23. Seagrave JC, Ramsey RC, Barker S, Martinez JR. Intracellular $[Ca^{2+}]_i$

and K^+ and Cl^- efflux responses in submandibular cells of neonatal and adult rats. Biochem Biophys Res Commun **188:**1161–1168, 1992.

24. Seagrave JC, Barker S, Martinez AM, Martinez JR. Muscarinic signaling pathway in submandibular cells of adult and early postnatal rats. Proc Soc Exp Biol Med **203:**490–499, 1993.
25. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem **260:**3440–3450, 1985.
26. Zhang GH, Melvin JE. Inhibitors of the intracellular Ca^{2+} release mechanism prevent muscarinic-induced Ca^{2+} influx in rat submandibular mucous acini. FEBS Lett **327:**1–6, 1993.
27. Zhang GH, Melvin JE. Membrane potential regulates Ca^{2+} uptake and inositol phosphate generation in rat sublingual mucous acini. Cell Calcium **14:**551–562, 1993.
28. Berridge MJ. Inositol trisphosphate and diacylglycerol: Two interacting second messengers. Annu Rev Biochem **56:**159–193, 1987.
29. Putney JW Jr., Bird GS. The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev **14:**610–631, 1993.
30. Baum BJ, Ambudkar IS, Horn VJ. Neurotransmitter control of calcium mobilization. In: Dobrosielski-Vergona K, Ed. Biology of the Salivary Glands. Boca Raton, FL: CRC Press, pp153–179, 1993.
31. Fasolato C, Zottini M, Clementi E, Zacchetti D, Meldolesi J, Pozzan T. Intracellular Ca^{2+} pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release. J Biol Chem **266:**20159–20167, 1991.
32. Gamberucci A, Fulceri R, Tarroni P, Giunti R, Marcolongo P, Sorrentino V, Benedetti A. Calcium pools in Ehrlich carcinoma cells. A major, high affinity Ca^{2+} pool is sensitive to both inositol 1,4,5-trisphosphate and thapsigargin. Cell Calcium **17:**431–441, 1995.
33. Thastrup O. Role of Ca^{2+}-ATPase in regulation of cellular Ca^{2+} signaling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. Agent Action **29:**8–15, 1990.
34. Martinez JR, Willis S, Puente S, Wells J, Helmke R, Zhang GH. Evidence for a Ca^{2+} pool associated with secretory granules in rat submandibular acinar cells. Biochem J **320:**627–634, 1996.
35. Merritt JE, Rink TJ. Rapid increases in cytosolic free calcium in response to muscarinic stimulation of rat parotid acinar cells. J Biol Chem **262:**4958–4960, 1987.
36. Nauntofte B, Dissing S. Stimulation-induced changes in cytosolic calcium in rat parotid acini. Am J Physiol **253:**G878–G886, 1987.
37. Putney JW Jr. Capacitative calcium entry revisited. Cell Calcium **11:**611–624, 1990.
38. Melvin JE, Koek L, Zhang GH. A capacitative Ca^{2+} influx is required for sustained fluid secretion in sublingual mucous acini. Am J Physiol **261:**G1043–G1050, 1991.
39. Xu X, Diaz J, Zhao H, Muallem S. Characterization, localization and axial distribution of Ca^{2+} signaling receptors in the rat submandibular salivary gland ducts. J Physiol **491:**647–662, 1995.
40. O'Connor SE, Dainty IA, Leff P. Further classification of ATP receptors based on agonist studies. TIPS **12:**137–141, 1991.
41. Jorgensen TD, Gromada J, Tritsaris K, Nauntofte B, Dissing S. Activation of P_{2z} purinoceptors diminishes the muscarinic cholinergic-induced release of inositol 1,4,5-trisphosphate and stored calcium in rat parotid acini. ATP as a co-transmitter in the stimulus-secretion coupling. Biochem J **312:**457–464, 1995.
42. Yu H, Turner JT. Functional studies in the human submandibular duct cell line, HSG-PA, suggest a second salivary gland receptor subtype for nucleotides. J Pharmacol Exp Ther **259:**1344–1350, 1991.
43. Dubyak GR. Purinergic signal transduction: Extracellular ATP and adenosine as agonists for receptor-mediated signaling. In: Brody JS, Center DM, Tkachuk VA, Eds. Signal Transduction in Lung Cells. New York: Marcel Dekker, pp199–223, 1993.
44. Hurley TW, Ryan MP, Shoemaker DD. Mobilization of Ca^{2+} influx, but not of stored Ca^{2+}, by extracellular ATP in rat submandibular gland acini. Archs Oral Biol **39:**205–212, 1994.
45. McMillian MK, Soltoff SP, Lechleiter JD, Cantley LC, Talamo BR. Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells. Differences from phospholipase C-linked receptor agonists. Biochem J **255:**191–300, 1988.
46. Soltoff SP, McMillian MK, Talamo BR. Coomassie brilliant blue G is a more potent antagonist of P_2 purinergic responses than reactive blue 2 (cibacron blue 3GA) in rat parotid acinar cells. Biochem Biophys Res Commun **165:**1279–1285, 1989.
47. Soltoff SP, McMillian MK, Talamo BR, Cantley LC. Blockage of ATP binding site of P_2 purinoceptors in rat parotid acinar cells by isothiocyanate compounds. Biochem Pharmacol **45:**1936–1940, 1993.
48. Horn VJ, Baum BJ, Ambudkar IS. β-Adrenergic receptor stimulation induces inositol trisphosphate production and Ca^{2+} mobilization in rat parotid acinar cells. J Biol Chem **263:**12454–12460, 1988.
49. Takemura H. Changes in cytosolic free calcium concentration in isolated rat parotid cells by cholinergic and β-adrenergic agonists. Biochem Biophys Res Commun **131:**1048–1055, 1985.
50. Doughney C, Dormer RL, McPherson MA. Adrenergic regulation of formation of inositol phosphates in rat submandibular acini. Biochem J **241:**705–709, 1987.
51. Hughes AR, Takemura H, Putney JW Jr. Does β-adrenoceptor activation stimulate Ca^{2+} mobilization and inositol trisphosphate formation in parotid acinar cells? Cell Calcium **10:**519–525, 1989.
52. Gromada J, Jorgensen TD, Dissing S. The release of intracellular Ca^{2+} in lacrimal acinar cells by α-, β-adrenergic and muscarinic cholinergic stimulation: The role of inositol trisphosphate and cyclic ADP-ribose. Pflugers Arch **429:**751–761, 1995.
53. Herlite S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits. Nature **380:**258–262, 1996.
54. De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP. Direct binding of G-protein $\beta\gamma$ complex to voltage-dependent calcium channels. Nature **385:**446–450, 1997.