Differences in Intracellular Ca^{2+} Stores of Submandibular Cells of Adult and Newborn Rats (44096)

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Abstract. **The intracellular Ca^{2+} stores of submandibular acinar cells of adult and newborn rats were compared by measuring changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) with the Ca^{2+}-sensitive fluorescent probe, fura-2, in intact cells and $^{45}Ca^{2+}$ release in permeabilized cells. In cells of adult rats, acetylcholine (ACh) and thapsigargin (TG) elicited, respectively, a 105% and 125% net increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium. In cells of newborn animals, the ACh-induced $[Ca^{2+}]_i$ increase (218%) was larger; but the TG-induced increase was significantly smaller (77%), suggesting that the TG-sensitive Ca^{2+} pump of the store is less developed, abundant, or responsive in these cells. After ACh or TG, ionomycin elicited a further Ca^{2+} release. Ionomycin by itself completely discharged the ACh- and TG-sensitive store in cells of mature glands but not in cells of immature glands, suggesting a further difference in the distribution of the agonist and ionomycin sensitivities in the two types of cells. After exposure to ACh + ionomycin or TG + ionomycin, collapsing the intracellular pH gradient with monensin caused a third and large Ca^{2+} release (262% and 289%, respectively) in cells of adult rats, but a significantly smaller release (96%, $P < 0.001$, and 57%, $P < 0.001$, respectively) in cells of newborn rats, suggesting that a third, IP_3-insensitive acidic store is significantly smaller in cells of immature glands. In permeabilized and $^{45}Ca^{2+}$-loaded cells, inositol-1,4,5-trisphosphate (IP_3, 5 μM) induced an identical $^{45}Ca^{2+}$ release (34% and 33%, respectively) in both types of cells, but TG induced a significant smaller $^{45}Ca^{2+}$ release in cells of immature glands (15%) than in cells of mature glands (33%). Monensin discharged <10% of loaded $^{45}Ca^{2+}$ in both types of cells, indicating that the radiotracer was not loaded into the monensin-sensitive store. These results suggest that in cells of immature glands: (1) the TG-sensitive Ca^{2+} pump of the IP_3-sensitive store is not well developed; (ii) the IP_3-sensitive store is not completely discharged by ionomycin; and (iii) an acidic store likely to be associated with secretory granules is also underdeveloped and of a smaller size than in cells of mature glands.**

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The inositol-1,4,5-triphosphate (IP_3)-Ca^{2+}–associated signaling system plays a critical role in the regulation of fluid and electrolyte secretion in salivary acinar cells. It is well recognized that fluid and electrolyte secretion in salivary glands is primarily regulated by the autonomic nervous system through the activation of muscarinic and α-adrenergic receptors (1–6). Stimulation of these receptors induces an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (7–11), which acts as a second messenger in the regulation of anion and K^+ channels and of the efflux of these ions which provides the driving force for secretion (12–14).

Recent studies (15–17) indicate that the signaling system of rat submandibular cells undergoes a unique maturation pattern during postnatal development (18). The generation of IP_3 stimulated by acetylcholine (ACh) in cells isolated from 1- and 7-day rats is significantly larger than that in cells of adult rats (17). Parallel to this response, the transient increase in $[Ca^{2+}]_i$ in response to muscarinic receptor stimulation is significantly larger in cells of immature submandibular glands (16, 17). However, the ATP-dependent $^{45}Ca^{2+}$ uptake and the IP_3-

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induced $^{45}Ca^{2+}$ release from non-mitochondrial stores measured in permeabilized cells are smaller in cells of immature glands (17). The reasons for this pattern of IP_3-Ca^{2+} signaling in cells of immature submandibular glands remain unclear.

The acetylcholine-induced initial increase in $[Ca^{2+}]_i$ in salivary acinar cells is due to release of Ca^{2+} from an IP_3-sensitive Ca^{2+} store and the subsequent sustained elevation is the result of Ca^{2+} influx from the extracellular fluid (7, 9, 11, 19). Re-uptake of Ca^{2+} into the IP_3-sensitive Ca^{2+} store occurs by way of an endoplasmic Ca^{2+}-ATPase (20–22) and extrusion of Ca^{2+} out of cells by the plasma membrane Ca^{2+}-ATPase. Both of these processes play critical roles in the regulation of $[Ca^{2+}]_i$ (22–25). There are therefore several possible mechanisms that could explain the observed difference seen in cells of immature rat submandibular glands between the large $[Ca^{2+}]_i$ signal in fura-2 studies and the small IP_3-induced $^{45}Ca^{2+}$ release seen in permeabilized cells. First, the Ca^{2+} pump responsible for the re-uptake of Ca^{2+} into the IP_3-sensitive Ca^{2+} store may be underdeveloped, resulting in more Ca^{2+} remaining in the cytosol after release from the store. Second, other intracellular Ca^{2+} stores besides the IP_3-sensitive one may also release Ca^{2+} in the cells of immature glands and contribute to the higher $[Ca^{2+}]_i$ seen after stimulation. Third, the plasma membrane Ca^{2+}-pump may be similarly underdeveloped, resulting in a slower and smaller Ca^{2+} extrusion and thus in a larger increase in $[Ca^{2+}]_i$ in response to stimulation. Fourth, intracellular Ca^{2+} buffering may not be as effective in cells of immature glands. The purpose of the present study was to explore the first two possibilities by comparing the characteristics of the intracellular Ca^{2+} stores present in submandibular cells of newborn and adult rats.

Materials and Methods

Animals. Male, 150- to 200-g Sprague-Dawley strain rats (Sasco Laboratories, Omaha, NB) and newborn (1-day-old) rats were used in all experiments. Adult rats were maintained in a room with controlled photoperiod (12:12-hr light:dark cycle) and temperature (23°C) and with standard rat chow and water available *ad libitum*.

Materials. Collagenase (type CLSPA) was from Worthington Biomedical (Malvern, PA). Acetylcholine, bovine serum albumin (BSA, type V), EGTA, gramicidin D, HEPES, hyaluronidase (type V), IP_3, ionomycin, monensin, and thapsigargin were from Sigma Chemical Co. (St. Louis, MO). Basal Medium Eagle Amino Acids (BME) was from GIBCO (Grand Island, NY). $^{45}CaCl_2$ was from NEN (Boston, MA). Fura-2/acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Eugene, OR). All other chemicals used were of the highest grade available.

Solutions. The digestion solution consisted of (in m*M*): 120 NaCl, 15 HEPES, 10 glucose, 4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1% (w/v) BSA, 1% (v/v) BME, 3 U/mg tissue of purified collagenase (type CLSPA; Worthington Biomedical), and 10 U/mg tissue of hyaluronidase. pH was adjusted to 7.4 with NaOH after gassing with O_2 at 37°C for 45 min. The Krebs-HEPES incubation solution (KHS) contained (in m*M*): 120 NaCl, 4 KCl, 15 HEPES, 10 glucose, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 1% BME amino acids, and 0.01% (w/v) BSA. pH was adjusted to 7.4 after gassing with O_2 for 45 min. $[Ca^{2+}]_i$ was determined using a Ca^{2+}-free KHS solution that contained the same components as KHS, except that Ca^{2+} was omitted and 20 μ*M* EGTA added. $^{45}Ca^{2+}$ uptake and release were measured in saponin-permeabilized cells in a cytosolic-like medium containing (in m*M*): 100 KCl, 20 NaCl, 15 glucose, 20 HEPES (pH 7.2), 5 $MgCl_2$, 0.02 EGTA (free Ca^{2+} 100 n*M*), 3 ATP, 10 phosphocreatine, 10 U/ml creatine phosphokinase, and 0.2% (w/v) BSA.

Preparation of Dispersed Submandibular Acini from Adult Rats. Submandibular acini from adult rats were isolated as previously described (15–17, 26, 27). Briefly, two rats were anesthetized with sodium pentobarbital (50 mg/kg), and the submandibular glands were excised, injected with 0.2 ml/gland of ice-cold digestion medium, dissected free of connective tissue, and rapidly minced in ice-cold digestion medium. The mince was incubated in an oxygenated dispersion medium (26) containing 3 U/mg wet tissue wt of purified collagenase and 10 U/mg wet tissue wt of hyaluronidase at 37°C in a shaking water bath for 60 min. The mince was dispersed by gently pipetting 10 times with a 10-ml plastic pipette at 10-min intervals during the last 30 min. After digestion for 60 min, the preparation was centrifuged at 50*g* for 2 min, and the supernatant was discarded. The preparation was resuspended in 10 ml of oxygenated washing medium and passed through a four-layer gauze and then washed twice by centrifugation at 50*g* for 2 min, and finally resuspended in fresh medium and kept at 23°C.

Isolation of Cell Aggregates from Newborns. Acinar cells of immature submandibular glands were isolated as previously described (15–17). Briefly, pregnant rats were obtained from Sasco Laboratories approximately 1 week before parturition. Newborn (1-day-old) rats were anesthetized with sodium pentobarbital (50 mg/kg wt). The submandibular glands were removed and placed in a digestion solution containing KHS, 1.4 U/mg tissue of collagenase, and 5 U/mg tissue of hyaluronidase. pH was adjusted to 7.4 after oxygenating for 45 min at 37°C. The submandibular tissues from newborn rats were then digested and collected as described above for cells from adult rats.

Digital Imaging of Intracellular Ca^{2+}. Isolated cell aggregates were loaded with fura-2 by incubation with

1 μ*M* fura-2/AM for 30 min at 23°C. Fura-2–loaded cells were attached to a glass coverslip and viewed using a QuantiCell digital imaging system (Applied Imaging, United Kingdom) at 23°C. Color-coded pictures were obtained using a Nikon Diaphot inverted microscope with objective Fluor 40×, NA 1.3 oil (Nikon).

Determination of $[Ca^{2+}]_i$ with a Dual-Wavelength Fluorometer. $[Ca^{2+}]_i$ determination was conducted as previously described (17, 28). Cells were loaded with the Ca^{2+}-sensitive fluorescent probe fura-2 by incubation with 1 μ*M* fura-2/AM for 30 min at 23°C in KHS (Ca^{2+}-containing) medium. Fura-2–loaded cells were rinsed twice with KHS containing 0.1% bovine serum albumin (BSA) and kept at 23°C. For measurement of $[Ca^{2-}]_i$, a 2-ml aliquot of fura-2–loaded cells was centrifuged at 50*g* for 2 min and resuspended in fresh, Ca^{2-}-free medium and placed in a 4-ml cuvette. $[Ca^{2+}]_i$ measurements were performed using a dual-wavelength PTI Deltascan 2.060a fluorometer (PTI Inc., S. Brunswick, NJ) at 37°C. 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 as previously described (17, 28). Briefly, 1 m*M* $CaCl_2$ and 50 μ*M* ionomycin were added 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 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 the method described by Grynkiewicz *et al.* (29) using 224 n*M* as K_d of fura-2 for Ca^{2+}.

Measurement of $^{45}Ca^{2+}$ Release in Permeabilized Cells. $^{45}Ca^{2+}$ release was determined as previously described (17). Isolated cell clusters were permeabilized by incubation in a cytosolic-like medium containing 45 μg/ml of saponin at 37°C for 10 min. The cells were then pelleted by centrifugation at 50*g* for 30 sec and resuspended in the same medium without saponin. An ATP-generation system consisting of 6 m*M* phosphocreatine and 8 U/ml of creatinine phosphokinase was used to maintain ATP concentration in the reaction system (30). The reaction mixture also contained the mitochondrial inhibitors oligomycin (10 μ*M*) and antimycin (10 μ*M*) (17). The cells were incubated with 1 μCi/ml $^{45}CaCl_2$ at 37°C for 2 min, then the ATP-dependent $^{45}Ca^{2+}$ uptake was initiated by addition of 3 m*M* ATP. The cells were incubated for 30 min until the cellular $^{45}Ca^{2+}$ content reached a steady state. $^{45}Ca^{2+}$ release was initiated by addition of Ca^{2+}-releasing agents. At 1, 2, 3, and 5 min after addition of the Ca^{2+}-releasing agents, $^{45}Ca^{2+}$ retained in the intracellular stores was determined by a rapid filtration technique (17, 31, 32). The filters containing cells were washed with 10 ml of ice-cold cytosolic-like medium, and $^{45}Ca^{2+}$ content measured by scintillation counting (LS3801, Beckman Instruments).

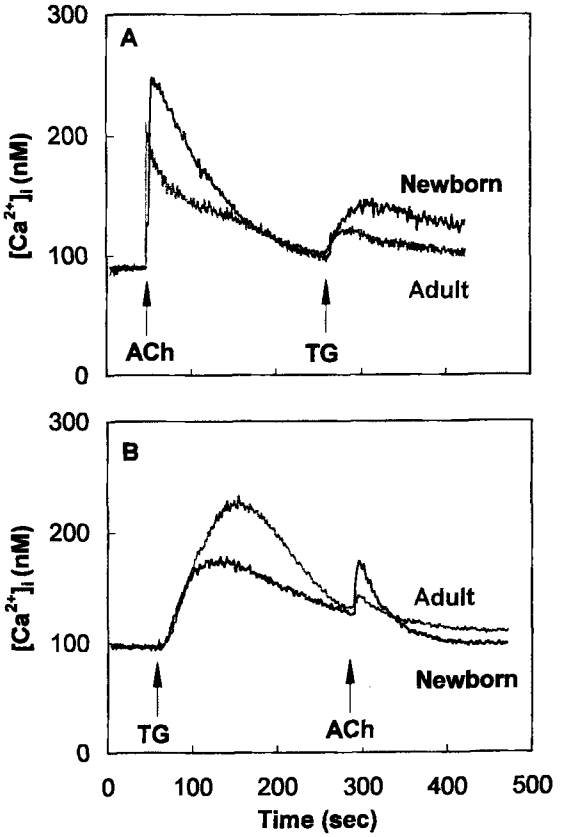

Figure 1. Ca^{2+} Release induced by acetylcholine and thapsigargin. Fura-2–loaded adult and newborn submandibular cells were suspended in 2 ml of Ca^{2+}-free medium and $[Ca^{2+}]_i$ measured by using a dual-wavelength fluorometer at 37°C. At the time indicated by the arrows the cells were stimulated with 1 μ*M* acetylcholine (ACh) followed by 3 μ*M* thapsigargin (TG) (A) or treated with TG first followed by ACh (B). Traces are representative of separate experiments using different cell prerparations (Panel A: adult, $n = 5$; newborn, $n = 7$; Panel B: adult, $n = 6$; newborn, $n = 4$).

Statistics and Data Presentation. All results are presented as mean ± SEM of at least four determinations using different cell preparations. Comparisons were made using the unpaired Student's *t* test. *P* values smaller than 0.05 were considered significant.

Results

ACh- and Thapsigargin-induced Ca^{2+} Release. As shown in Figure 1A, stimulation with ACh (1 μ*M*) induced a 104.7% ± 10.1% increase in $[Ca^{2+}]_i$ in cells of adult rats (from 92.8 ± 2.3 n*M* to 189.8 ± 10.1 n*M*; $n = 5$). In cells of newborn rats, the same treatment induced a 217.5% ± 39.7% increase in $[Ca^{2+}]_i$ (from 72.0 ± 3.9 n*M* to 234.6 ± 40.0 n*M*; $n = 7$). This increase was significantly larger than that seen in cells of adult rats ($P < 0.05$). These results are consistent with previous reports (16, 17). On the other hand, the endoplasmic Ca^{2-}-ATPase inhibitor TG (3 μ*M*) induced a 124.5% ± 15.8% increase in $[Ca^{2+}]_i$ in cells of adult rats (from 92.2 ± 5.4 n*M* to 210.2 ± 7.9 n*M*; $n = 6$; Fig. 1B), but a significantly smaller increase in cells of newborn rats

(76.8% ± 16.5%; from 83.0 ± 8.9 n*M* to 144.3 ± 13.1 n*M*; $n = 4$; $P < 0.05$; Fig. 1B).

In cells of adult animals, the IP_3-sensitive Ca^{2+} store can be essentially discharged by either the stimulation of muscarinic receptors or inhibition of the endoplasmic Ca^{2+} pump with TG (11, 21, 28). In cells of immature glands, stimulation of muscarinic receptors induced a larger increase in $[Ca^{2+}]_i$, but inhibition of the endoplasmic Ca^{2+} pump caused a significantly smaller Ca^{2+} release. A possible explanation is that the distribution of IP_3 and TG sensitivity overlaps in cells of mature glands but not in cells of immature glands. To examine this possibility, cells from adult and newborn animals were exposed sequentially to TG and ACh. Treatment with TG (3 μ*M*) after ACh (1 μ*M*) caused a 19.4% ± 4.3% further increase in $[Ca^{2+}]_i$ in adult cells ($n = 5$) and a 41.5% ± 6.8% further increase in cells of newborn rats ($n = 7$; $P < 0.05$; Fig. 1A). In the opposite sequence of stimulation, a subsequent exposure to ACh after TG induced a 10.9% ± 2.4% further increase in $[Ca^{2+}]_i$ in cells of adult rats ($n = 6$) and a 57.8% ± 12.7% further increase in cells of newborn rats ($n = 4$; $P < 0.005$; Fig. 1B).

Ionomycin-Induced Ca^{2+} Release. In a previous study (28), we found that exposure of adult rat submandibular cells to ionomycin induced a relatively large Ca^{2+} release and that the subsequent stimulation with ACh or TG did not induce any further release. In the present study, exposure to ionomycin (1 μ*M*) induced a 170.5% ± 13.5% increase in $[Ca^{2+}]_i$ (from 109.8 ± 7.5 n*M* to 298.8 ± 29.4 n*M*; $n = 6$; Fig. 2) in cells isolated from adult rats. This treatment completely emptied the agonist-sensitive Ca^{2+} store, since a subsequent exposure to ACh or to TG did not induce any further increase in $[Ca^{2+}]_i$ (Fig. 2). In cells of newborn animals, by contrast, 1 μ*M* ionomycin caused a 120.4% ± 8.2% increase in $[Ca^{2+}]_i$ (from 76.1 ± 2.4 n*M* to 167.4 ± 6.4 n*M*; $n = 7$; Fig. 2), a response which was significantly smaller than that seen in cells of adult rats ($P < 0.01$); and a subsequent stimulation with ACh or TG induced a 77.8% ± 7.8% ($n = 7$) and 130.9% ± 10.6% ($n = 7$) further increase in $[Ca^{2+}]_i$, respectively (Fig. 2). These results suggest that, in contrast to what is seen in cells of adult rats, a portion of the IP_3-sensitive and TG-sensitive Ca^{2+} stores in cells of immature glands is insensitive to ionomycin.

In the previous study (28), we found that after the IP_3-sensitive store is emptied, the subsequent exposure to ionomycin elicited a further Ca^{2+} release from an IP_3-insensitive but ionomycin-sensitive store. To further explore the relationship between the IP_3-sensitive store and the IP_3-insensitive but ionomycin-sensitive store, cells were treated with ACh or TG first, followed by ionomycin. After stimulation with ACh (1 μ*M*), ionomycin (1 μ*M*) caused a 40.9% ± 6.0% ($n = 13$) and 54.4% ± 9.4% ($n = 8$) further increase in $[Ca^{2+}]_i$ in cells

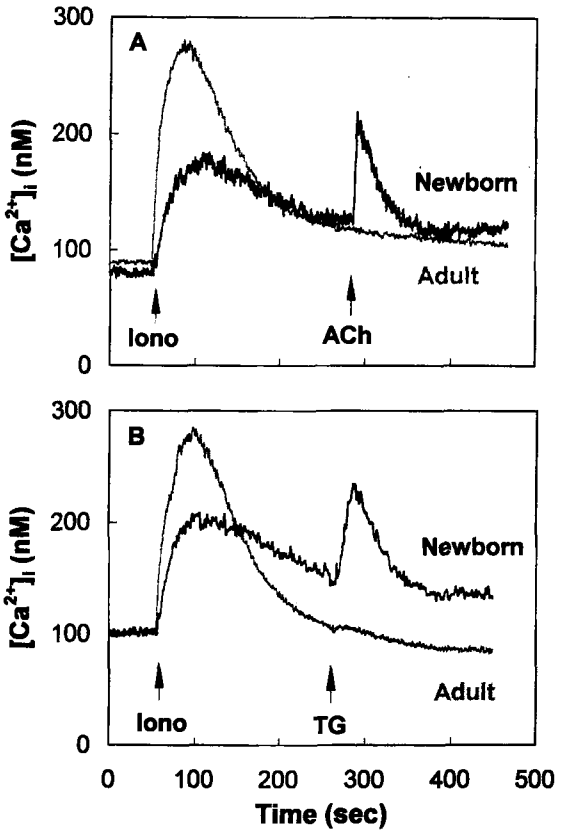

Figure 2. Effect of ionomycin on the acetylcholine- and thapsigargin-induced Ca^{2+} release. Fura-2–loaded submandibular cells of adult and newborn rats were treated with 1 μ*M* ionomycin (Iono) followed by 1 μ*M* acetylcholine (ACh) (A) or 3 μ*M* thapsigargin (TG) (B). Traces are representative of separate experiments using different cell preparations (Panel A: adult, $n = 6$; newborn, $n = 7$; Panel B: adult, $n = 6$; newborn, $n = 7$).

of adult and newborn rats, respectively (Fig. 3A), while treatment with 1 μ*M* ionomycin after TG caused a 51.5% ± 7.2% ($n = 7$) and 49.4% ± 14.2% ($n = 9$) further increase in $[Ca^{2+}]_i$ in the two types of cells (Fig. 3B). In general, therefore, the ionomycin-induced increases in $[Ca^{2+}]_i$ after TG or ACh were not significantly different between cells of mature and immature glands.

Ca^{2+} Release from an Acidic Ca^{2+} Store. Many cell types have slowly exchanging Ca^{2+} stores usually associated with acidic organelles (33, 34). Under physiological conditions, $^{45}Ca^{2+}$ uptake into these stores is slow, requiring hours or days to reach isotopic equilibrium (34), and Ca^{2+} ionophores, such as ionomycin or A23187, are not effective in such acidic environments (33, 35), so that they discharge only a small fraction of Ca^{2+} from such stores. In order to fully empty these stores, a combination of a Ca^{2+} ionophore and a reagent that collapses the pH gradient across the organelle membrane is required (35). In the previous study (28), we found evidence that a large acidic pool of this type is present in submandibular acinar cells of adult rats. To examine whether a similar pool is also present and functional in cells of immature glands, we measured Ca^{2+}

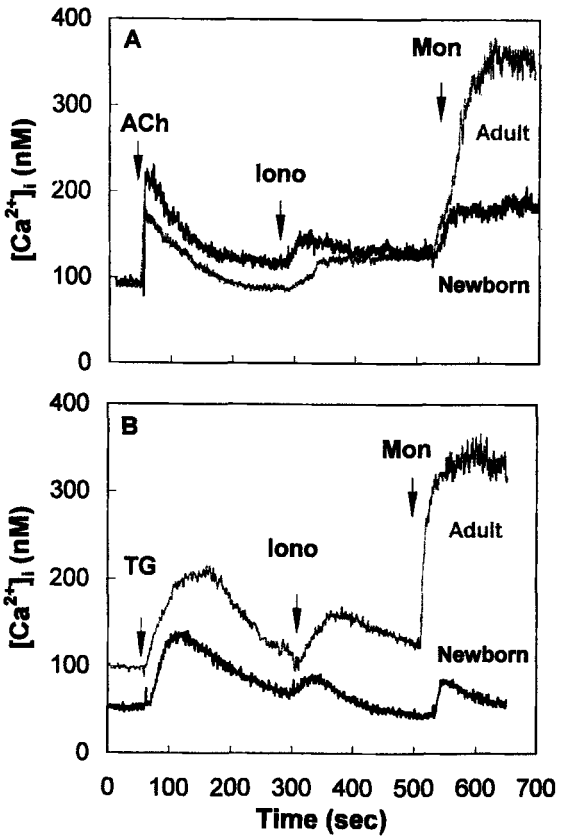

Figure 3. $[Ca^{2+}]_i$ changes induced by sequential treatment with acetylcholine or thapsigargin, ionomycin, and monensin. Fura-2–loaded adult and newborn cells were sequentially treated with 1 μM acetylcholine (ACh), 1 μM ionomycin (Iono), and 10 μM monensin (Mon) (A) or with 3 μM thapsigargin (TG), 1 μM ionomycin, and 10 μM monensin (B). Traces are representative of separate experiments using different cell preparations (Panel A: adult, $n = 7$; newborn, $n = 8$; Panel B: adult, $n = 7$; newborn, $n = 8$).

release induced by a combination of ionomycin and the Na^+/H^+ ionophore monensin. Exposure of the cells of adult rats to monensin (10 μM), after emptying the IP_3- and ionomycin-sensitive stores with ACh and ionomycin, induced a large third increase in $[Ca^{2+}]_i$ (262.4% ± 36.2%; $n = 7$; Fig. 3A). In cells of newborn rats, however, monensin exposure after ACh + ionomycin only induced a 96.1% ± 15.0% increase in $[Ca^{2+}]_i$ ($n = 8$; $P < 0.001$ compared with adult; Fig. 3A). In the same fashion, exposure to monensin after TG + ionomycin induced a 288.7% ± 38.8% further increase in $[Ca^{2+}]_i$ in cells of adult rats ($n = 7$; Fig. 3B) and a 57.3% ± 8.4% further increase in cells of newborn rats ($n = 8$; $P < 0.001$; Fig. 3B), suggesting that the acidic pool in cells of immature glands is not as large or well developed as that of cells of mature glands.

To confirm the observation shown in Figure 3, changes in $[Ca^{2+}]_i$ were monitored using the digital imaging system (Fig. 4). Stimulation with ACh triggered a 122% net increase in $[Ca^{2+}]_i$ in cells of adult rats (from 73.6 ± 6 nM to 164.1 ± 7.9 nM; $n = 5$; Fig. 4B) and a 164% increase in cells of newborn rats (from 85.5 ± 3.5 nM to 225.5 ± 15.2 nM; $n = 8$; $P < 0.05$; Fig. 4F). Subsequent exposure to ionomycin (1 μM) caused a 92% further increase in $[Ca^{2+}]_i$ in cells of adult glands (Fig. 4C), but no increase was observed in cells of newborn glands ($P < 0.01$; Fig. 4G). After ionomycin, a subsequent exposure to monensin (10 μM) after the other two drugs resulted in an additional 14% increase in $[Ca^{2+}]_i$ of cells of newborn rats (Fig. 4H), while a large increase (247%) was triggered in cells of adult glands ($P < 0.001$; Fig. 4D). In a similar fashion, exposure to TG (3 μM) elicited a 148% increase in $[Ca^{2+}]_i$ in cells of adult (from 100.5 ± 8 nM to 248.9 ± 10.2 nM; $n = 5$) and 99% increase in cells of newborn rats (from 95.9 ± 6.1 nM to 190.6 ± 17.3 nM; $n = 4$; $P < 0.05$). Subsequent exposure to ionomycin induced a 143% increase in cells of adult rats, and only 16% increase in cells of newborn rats ($P < 0.01$). A subsequent exposure to monensin caused a 232% increase in cells of adult rats, but only a 20% increase in cells of newborn rats ($P < 0.005$).

In cells isolated from mature glands, the Ca^{2+} release induced by ionomycin + monensin is from a large acidic storage site which is a slowly exchanging store and is thought to be associated with secretory granules (28). It is likely, therefore, that the much smaller release of Ca^{2+} observed under these conditions in the cells of newborn rats is related to the lack of mature secretory granules (36). To confirm this hypothesis, the release of $^{45}Ca^{2+}$ was compared in permeabilized cells. In cells of adult rats, IP_3 (5 μM; Fig. 5A) and TG (3 μM; Fig. 5B) induced a 34.1% ± 3.6% ($n = 7$) and 33.7% ± 0.9% ($n = 3$) release, respectively, of the $^{45}Ca^{2+}$ content in 5 min. In cells of newborn rats, IP_3 elicited a 33.0% ± 7.0% ($n = 3$) $^{45}Ca^{2+}$ release (Fig. 5A), but TG caused only a 15.0% ± 3.0% ($n = 3$; $P < 0.005$ compared with adult) $^{45}Ca^{2+}$ release (Fig. 5B). After IP_3 or TG, ionomycin (1 μM) induced a 49.8% ± 8.2% ($n = 4$; Fig. 5A) and a 46.3% ± 7.1% ($n = 4$; Fig. 5B) further $^{45}Ca^{2+}$ release in cells of adult rats, respectively. In cells of newborn rats, the ionomycin-induced $^{45}Ca^{2+}$ release was 52.0% ± 8.0% ($n = 3$; Fig. 5A) and 60.3% ± 4.1% ($n = 3$; Fig. 5B) after ACh and TG, respectively. Obviously, the larger $^{45}Ca^{2+}$ release induced by ionomycin in TG-pretreated cells of newborn animals is because TG discharged less $^{45}Ca^{2+}$ and the residual $^{45}Ca^{2+}$ was released by ionomycin. After IP_3 + ionomycin or TG + ionomycin, monensin induced a small $^{45}Ca^{2+}$ release (<10%) in the two types of cells (Fig. 5). These results show that during a 30-min loading, only a small amount of isotope was taken up into the acidic pool. This is consistent with the characteristics of slowly exchanging Ca^{2+} stores (SECS).

Discussion

Previous studies demonstrated that, compared with cells from adult animals, cells isolated from the subman-

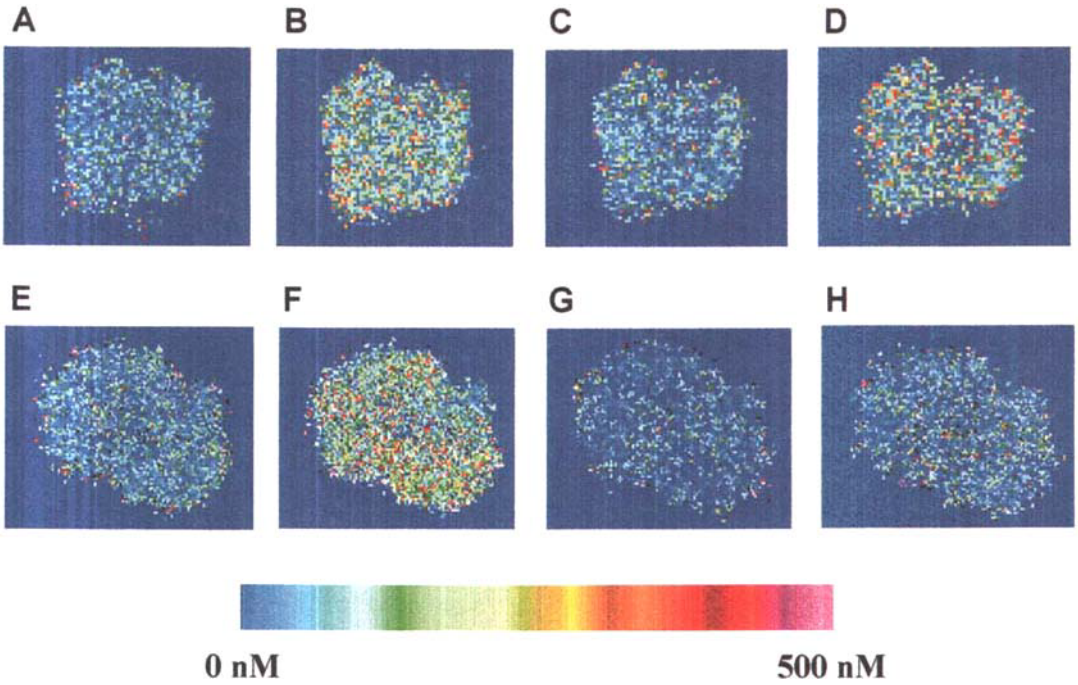

Figure 4. Digital imaging of cytosolic free Ca^{2+} changes. Fura-2–loaded cells were perfused with a Ca^{2+}-free medium at 23°C (A and E), then sequentially treated with acetylcholine (B and F), ionomycin (C and G), and monensin (D and H). Panels A–D are for submandibular cells of adult rats, and Panels E–H for cells of newborn rats. The color reference under the imaging pictures shows the relationship between fluorescence intensity (or $[Ca^{2+}]_i$) and color. Elevation in fluorescence intensity (or $[Ca^{2+}]_i$) is presented as a change in color from blue to magenta. Pictures are representative of separate experiments (adult, $n = 6$; newborn, $n = 8$).

dibular salivary gland of newborn and early postnatal rats show a large ACh-induced increase in $[Ca^{2+}]_i$ when the response was monitored with fura-2 but a smaller ATP-dependent uptake and a smaller IP_3-induced release of Ca^{2+} when the response was investigated using the radiotracer $^{45}Ca^{2+}$ in permeabilized cells (16–18). We speculated that differences in the distribution or functional characteristics of the intracellular Ca^{2+} stores may be among the reasons for the discrepancy in these responses and for the differences between cells of mature and immature glands. The results of this study indeed show important differences in both the IP_3-sensitive store discharged by stimulation with agonist- and an IP_3-insensitive acidic store, previously shown to be present in mature submandibular acini (28).

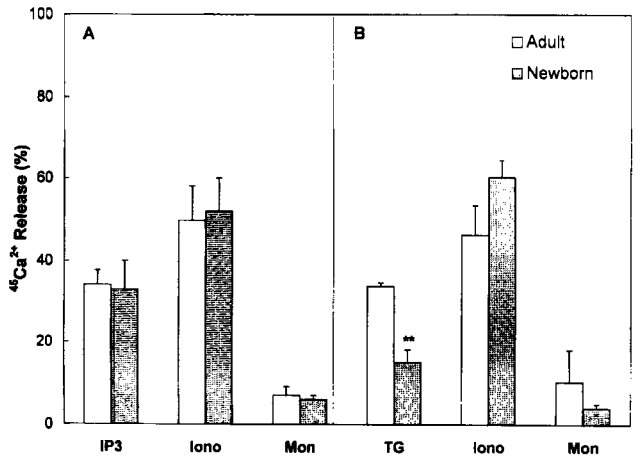

Figure 5. Ca^{2+} release from permeabilized cells. Submandibular cells of adult and newborn rats were permeabilized with saponin and loaded with $^{45}Ca^{2+}$ for 30 min at 37°C in a cytosolic-like medium containing mitochondrial inhibitors and an ATP regeneration system. $^{45}Ca^{2+}$ release was initiated by sequentially adding 5 μM inositol-1,4,5-trisphosphate (IP_3) or 3 μM thapsigargin (TG), followed by 1 μM ionomycin (Iono) and 10 μM monensin (Mon). Values presented are the $^{45}Ca^{2+}$ released at 5 min after addition of each agent. Data are presented as mean ± SEM of at least three separate experiments. **Compared with adult, $P < 0.005$.

Cells of both immature and mature submandibular glands have an agonist- and IP_3-sensitive Ca^{2+} store of approximately the same capacity, as shown by the similar extent of $^{45}Ca^{2+}$ release induced by IP_3 in both types of cells (Fig. 5). This store is also sensitive to the specific inhibitor of the endoplasmic Ca^{2+} pump, TG (9, 21, 37, 38), and the Ca^{2+} released by ACh and by TG presumably originates in the same IP_3-sensitive store in salivary acinar cells (28, 39). An important difference, however, in the cells of immature glands is that the TG-induced Ca^{2+} release was significantly smaller than that in cells of mature glands. This suggests that the ATP-dependent and TG-sensitive Ca^{2+} uptake into the store is not as well developed in the cells of early postnatal animals. Since TG acts by inhibiting the Ca^{2+}-ATPase in the store-limiting membrane, the simplest explanation of this finding is that the Ca^{2+}-ATPase is either not present in the same density or is less responsive in the store of the cells of immature glands. This is supported by the

observation that exposure of these cells to ACh following a prior TG exposure caused a considerable additional Ca^{2+} release (Fig. 1), and by a previous observation that ATP-dependent $^{45}Ca^{2+}$ uptake was smaller in permeabilized cells of immature glands (17). Underdevelopment or immaturity of the Ca^{2+}-ATPase in cells of immature glands would result in less re-uptake of Ca^{2+} after IP_3-induced release and could explain, at least partially, the larger increase in $[Ca^{2+}]_i$ previously observed in these cells following ACh stimulation in experiments using fura-2 (17). An alternative explanation is that transport mechanisms other than a Ca^{2+} pump may play a role in Ca^{2+} sequestration into the store of cells derived from immature glands.

Recent evidence indicates that the agonist- and thapsigargin-sensitive Ca^{2+} release do not completely overlap in submandibular cells of adult rats, as the thapsigargin-induced Ca^{2+} release is larger than that induced by agonist and a further release of Ca^{2+} is seen, furthermore, in cells exposed to thapsigargin following exposure to agonist (28). The same is seen in other cell types such as Jurkat T lymphocytes (40) and PANC-1 cells (41), where the thapsigargin-induced Ca^{2+} release is larger than that induced by IP_3. Our results in this study show that this is also true in the cells of immature glands used in this study (Fig. 1). A second difference in the IP_3-sensitive Ca^{2+} store of the cells of immature glands is that the IP_3 and TG sensitivities do not overlap as completely as they do in cells of mature glands. In cells of mature submandibular glands, inhibition of the Ca^{2+} pump with TG discharged the IP_3-sensitive store, and a subsequent exposure to ACh could not elicit any substantial Ca^{2+} release. However, in cells of immature glands, after TG treatment, a subsequent ACh stimulation still caused a significant Ca^{2+} release. It is possible that the IP_3 sensitivity (i.e., the IP_3-sensitive Ca^{2+} channels) and the TG sensitivity (i.e., the Ca^{2+} pumps) have a separate distribution in the cells of the immature glands and that these distributions merge during development. Further investigation on this possibility is required.

The third difference in the IP_3-sensitive Ca^{2+} store of cells of immature glands is the incomplete release of Ca^{2+} caused from it by ionomycin. This ionophore can completely discharge the ACh- and TG-sensitive Ca^{2+} pools in acinar cells of adult rats (28). In cells of immature glands, by contrast, there was a further significant Ca^{2+} release when cells previously exposed to ionomycin were exposed to ACh or TG. Since the release of Ca^{2+} induced by ionomycin alone was smaller in cells of immature glands (Fig. 2), it appears that the IP_3-sensitive store of these cells is less sensitive to ionomycin. The reason for this lower sensitivity is not clear but could be explained if the store in the cells of newborn rats is more acidic than the store in mature cells, since ionomycin is not capable of discharging Ca^{2+} from this type of store (34).

The effect of ionomycin on Ca^{2+} release seen in the sequential drug exposure with the digital imaging system (Fig. 4) was significantly larger than that seen in the fura-2 fluorescence experiment (Fig. 3). In addition, the response of cells of adult rats in the imaging experiments was larger than in cells of newborn rats (Fig. 4), while this was not the case in the fura-2 studies (Fig. 3). The reason for this difference is not clear but may be related to the number of cells involved in each procedure or to differences in experimental conditions in the two type of experiments. Only a few cells or an acinus are used in the imaging studies, while a large number of cells are used in the fura-2 studies. Imaging experiments were performed at 23°C, while the fura-2 experiments were conducted at 37°C. The matter is under further investigation, but it is important to note that the responses to other substances (ACh, TG, monensin) were quite comparable in the two experimental approaches.

We recently demonstrated that cells of mature submandibular gland have a large IP_3-insensitive and probably acidic Ca^{2+} store which releases Ca^{2+} when exposed to ionomycin and to a substance like monensin which collapses the pH gradient (28). This store is associated with secretory granules and probably represents a slowly exchanging Ca^{2+} store. Ca^{2+} uptake into the store is slow and does not seem to involve the Ca^{2+} pump, which is the major Ca^{2+}-sequestering mechanism in the IP_3-sensitive Ca^{2+} store. Our results show that the capacity of this store is considerably smaller in cells of immature glands (Figs. 3 and 4). Two important characteristics in secretory granules and secretory proteins may underlie this difference in the IP_3-insensitive Ca^{2+} store of the two cell populations. One is that the submandibular cells of newborn rats have few secretory granules (42–44). The submandibular gland of newborn rats has two transient secretory cell types, type I and type III (45), which are capable of secreting the granular content in response to, respectively, cholinergic and β-adrenergic stimulation (45, 46). However, the small number of granules in these cells can limit their capacity for Ca^{2+} storage in this compartment. Even if the few secretory granules present in type I and type III cells were enough to sequester Ca^{2+}, the granular material of the adult submandibular gland, mucin (47) and glutamic acid-rich protein (GRP) (48), would be present in very small amount in type I and type III cells (49). The contents of the granules in these cells may not have the same affinity for Ca^{2+} as adult material, and this would limit the capacity for Ca^{2+} storage. The view that it is the relative lack of secretory granules which explains the small capacity of the ionomycin + monensin–sensitive pool in the cells of newborn animals is supported by the observation that in cells of adult rats degranulation

by isoproterenol abolishes the Ca^{2+} response to these drugs (28).

In summary, the present study shows differences in the functional characteristics of intracellular Ca^{2+} stores in cells of mature and immature submandibular cells. Cells of immature glands release less Ca^{2+} from an IP_3-insensitive and acidic store in secretory granules and show less sensitivity to ionomycin and less Ca^{2+} re-uptake in the IP_3-sensitive Ca^{2+} pool. The larger Ca^{2+} signal induced by ACh in cells of immature glands in Figure 3 is therefore not due to enhanced release from the IP_3-sensitive store or from other stores, but is related, at least in part, to a reduced Ca^{2+} re-uptake into the IP_3-sensitive store as a result of smaller density or functional integrity of Ca^{2+} pumps. An additional contributing factor may be less Ca^{2+} extrusion by the plasma membrane Ca^{2+}-ATPase if this transport system is also underdeveloped in cells of immature glands. This is currently under investigation.

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1. Baum BJ. Neurotransmitter control of secretion. J Dent Res **66:**628–632, 1987.
2. Martinez JR. Ion transport and water movement. J Dent Res **66:**638–647, 1987.
3. Yu JH. The influence of varying the electrical frequency of sympathetic nerve stimulation on fluid and calcium secretion of the rat parotid salivary gland. Arch Oral Biol **35:**639–643, 1990.
4. Yu JH, Redman RS. Effects of clonidine on the calcium content and morphology of rat salivary glands. Arch Oral Biol **37:**1067–1078, 1992.
5. Yu JH, Mark MR, Redman RS. Effect of clonidine on secretion of fluid and ions by the parotid and submandibular glands of the rat. Arch Oral Biol **32:**27–33, 1987.
6. Melvin JE, Zhang GH. Altered responses to agonists after chronic in vivo atropine administration in rat parotid acini. Crit Rev Oral Biol Med **4:**427–434, 1993.
7. Nauntofte B, Dissing S. Stimulation-induced changes in cytosolic calcium in rat parotid acini. Am J Physiol **253:**G878–G886, 1987.
8. Putney JW Jr. Stimulus-permeability coupling: Role of calcium in the receptor regulation of membrane permeability. Pharmac Rev **30:**209–245, 1979.
9. Putney JW Jr., Bird GS. The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev **14:**610–631, 1993.
10. Ambudkar IS, Melvin JE, Baum BJ. α_1-Adrenergic regulation of Cl^- and Ca^{2+} movements in rat parotid acinar cells. Pfluegers Arch **412:**75–79, 1988.
11. Melvin JE, Koek L, Zhang GH. A capcitative Ca^{2+} influx is required for sustained fluid secretion in sublingual mucous acini. Am J Physiol **261:**G1043–G1050, 1991.
12. Martinez JR, Cassity N. ^{36}Cl fluxes in dispersed rat submandibular acini: Effects of Ca^{2+} omission and of the ionophore A23187. Pfluegers Arch **407:**615–619, 1986.
13. Maruyama Y, Gallacher DV, Petersen OH. Voltage and Ca^{2+} activated K^+ channel in basolateral acinar cell membrane of mammalian salivary glands. Nature **302:**827–829, 1983.
14. Nauntofte B, Poulsen JH. Effects of Ca^{2+} and furosemide on Cl^- transport and O_2 uptake in parotid acini. Am J Physiol **251:**C175–C185, 1986.
15. Martinez JR, Camden J. Ca^{2+} mobilization and Cl^- efflux in submandibular salivary cells of adult and newborn rats. Arch Oral Biol **34:**147–152, 1989.
16. Seagrave JC, Ramsey RC, Barker S, Martinez JR. Intracellular Ca^{2+} and K^+ and Cl^- efflux responses in submandibular cells of neonatal and adult rats. Biochem Biophys Res Commun **188:**1161–1168, 1992.
17. Seagrave JC, Barker S, Martinez M, Martinez JR. Muscarinic signaling pathway in submandibular cells of adult and early postnatal rats. Proc Soc Exp Biol Med **203:**490–500, 1993.
18. Martinez JR. Developmental aspects of fluid and electrolyte secretion in salivary glands. Crit Rev Oral Biol Med **5:**281–290, 1994.
19. 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.
20. Petersen CC, Petersen OH, Berridge MJ. The role of endoplasmic reticulum calcium pumps during cytosolic calcium spiking in pancreatic acinar cells. J Biol Chem **268:**22262–22264, 1993.
21. 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.
22. Carafoli E. The Ca^{2+} pump of the plasma membrane. J Biol Chem **267:**2115–2118, 1992.
23. Zhang B-X, Zhao H, Loessberg P, Muallem S. Activation of the plasma membrane Ca^{2+} pump during agonist stimulation of pancreatic acini. J Biol Chem **267:**15419–15425, 1992.
24. Tepikin AV, Voronina SG, Gallacher DV, Petersen OH. Acetylcholine-evoked increase in the cytoplasmic Ca^{2+} concentration and Ca^{2+} extrusion measured simultaneously in single mouse pancreatic acinar cells. J Biol Chem **267:**3569–3572, 1992.
25. Tepikin AV, Voronina SG, Gallacher DV, Petersen OH. Pulsatile Ca^{2+} extrusion from single pancreatic acinar cells during receptor-activated cytosolic Ca^{2+} spiking. J Biol Chem **267:**14073–14076, 1992.
26. Seagrave JC, Barker S, Curry M, Martinez JR. Effects of NH_4Cl and dimethylamine on Cl^- fluxes in resting and stimulated rat submandibular acinar cells. Am J Physiol **263:**G558–G565, 1992.
27. Martinez JR, Camden J, Barker S. Effects of acetylcholine and monensin on ^{22}Na uptake and cytosolic Ca^{2+} in rat submandibular salivary cells. Archs Oral Biol **35:**359–364, 1990.
28. 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.
29. 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.
30. Burgess GM, Irvine RF, Berridge MJ, McKinney JS, Putney JW Jr. Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes. Biochem J **224:**741–746, 1984.
31. Zhang GH, Yamaguchi M, Kimura S, Higham S, Kraus-Friedmann N. Effects of heavy metal on rat liver microsomal Ca^{2+}-ATPase and Ca^{2+} sequestering. Relation to SH groups. J Biol Chem **265:**2184–2189, 1990.
32. Zhang GH, Cragoe EJ Jr., Melvin JE. Na^+ influx is mediated by Na^+-K^+-$2Cl^-$ cotransport and Na^+-H^+ exchange in sublingual mucous acini. Am J Physiol **264:**C54–C62, 1993.
33. Shorte SL, Collingridge GL, Randall AD, Chappell JB, Schofield JG. Ammonium ions mobilize calcium from an internal pool which is insensitive to TRH and ionomycin in bovine anterior pituitary cells. Cell Calcium **12:**301–312, 1991.
34. Possan T, Rizzuto R, Volpe P, Meldolesi J. Molecular and cellular physiology of intracellular calcium stores. Physiol Rev **74:**595–636, 1994.
35. Pressman BC, Fahim M. Pharmacology and toxicology of the monovalent carboxylic ionophores. Annu Rev Pharmacol Toxicol **22:**465–490, 1982.

36. Ball WD. Cell-restricted secretory proteins as markers of cellular phenotype in salivary glands. In: Dobrosielski-Vergona K, Ed. Biology of the Salivary Glands. Boca Raton, FL: CRC Press, pp 355–395, 1993.
37. 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.
38. 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.
39. Zhang GH, Melvin JE. Inhibitors of the intracellular Ca^{2+} release mechanism prevent muscarinic-induced Ca^{2+} influx in rat sublingual mucous acini. FEBS Lett **327:**1–6, 1993.
40. Guse AH, Roth E, Emmrich F. Intracellular Ca^{2+} pools in Jurkat T-lymphocytes. Biochem J **291:**447–451, 1993.
41. Wu L, Katz S, Brown GR. Inositol 1,4,5-trisphosphate-, GTP-, arachidonic acid- and thapsigargin-mediated intracellular calcium movement in PANC-1 microsomes. Cell Calcium **15:**228–240, 1994.
42. Jacoby F, Leeson C. The postnatal development of the rat submaxillary gland. J Anat **93:**201–216, 1959.
43. Gresik EW. Postnatal developmental changes in submandibular glands of rats and mice. J Histochem Cytochem **28:**860–870, 1980.
44. Martinez JR, Camden J. ^{36}Cl fluxes in dispersed-cell aggregates of the submandibular salivary gland of early postnatal rats. Arch Oral Biol **32:**659–663, 1987.
45. Ball WD, Redman RS. Two independently regulated secretory systems within the acini of the submandibular gland of the perinatal rat. Eur J Cell Biol **33:**112–122, 1984.
46. Cutler LS, Christian CP, Bottaro B. Development of stimulus-secretion coupling in salivary glands. In: Hand AR, Oliver C, Eds. Methods in Cell Biology. New York: Academic Press, pp 531–545, 1981.
47. Tabak LA, Mirels L, Monte LD, Ridall AL, Levine MJ, Loomis RE, Lindauer F, Reddy MS, Baum BJ. Isolation and characterization of a mucin-glycoprotein from rat submandibular glands. Arch Biochem Biophys **242:**383–392, 1985.
48. Mirels L, Bedi GS, Dickinson DP, Gross KW, Tabak LA. Molecular characterization of glutamic acid/glutamine-rich secretory proteins from rat submandibular glands. J Biol Chem **262:**7289–7297, 1987.
49. Moreira JE, Ball WD, Mirels L, Hand AR. Accumulation and localization of two adult acinar cell secretory proteins during development of the rat submandibular gland. Am J Anat **191:**167–184, 1991.