Evidence for a Ca^{2+} pool associated with secretory granules in rat submandibular acinar cells

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Intracellular Ca^{2+} stores in rat submandibular acinar cells were characterized using the Ca^{2+} -sensitive fluorescent indicator fura 2 and the radiotracer $^{45}\text{Ca}^{2+}$. Acetylcholine induced a rapid Ca^{2+} release from a store sensitive to inositol 1,4,5-trisphosphate (IP_3) and to thapsigargin (TG). After this store was presumably depleted, ionomycin caused a further increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), suggesting the presence of an IP_3 -insensitive Ca^{2+} release from a store that is more extensive and heterogeneous than the IP_3 -sensitive one and includes a small mitochondrial component. After both of these stores had been discharged, exposure to monensin caused an additional release of Ca^{2+} from a third store. This store appears to be associated with secretory granules, since Ca^{2+} release was significantly reduced when degranulation was induced by isoprenaline. This third store appears to be insensitive to IP_3 , discharges Ca^{2+} when the pH gradient across the limiting membrane is collapsed with

monensin and only in the presence of both ionomycin and monensin. Ca^{2+} release from this store is not by $\text{Na}^+/\text{Ca}^{2+}$ exchange, since simply altering $[\text{Na}^+]_i$ did not cause significant Ca^{2+} release. In permeabilized cells, IP_3 and TG released approx. 35 % of $^{45}\text{Ca}^{2+}$, and ionomycin released an additional 57 %, whereas monensin only caused a small additional release, suggesting that only IP_3 - and ionomycin-sensitive stores are loaded with $^{45}\text{Ca}^{2+}$ under these conditions. The absence of significant isotope uptake into the ionomycin + monensin-sensitive store may result from a low rate of tracer accumulation or from the lack of Ca^{2+} pumps in the store. The pattern of response was similar in the presence and absence of mitochondrial inhibitors, indicating that the store is not located in mitochondria. In summary, these results suggest that a substantial IP_3 -insensitive Ca^{2+} store is present in secretory granules in rat submandibular acinar cells.

INTRODUCTION

Fluid and electrolyte secretion in salivary acinar cells is mainly regulated by cholinergic and α_1 -adrenergic stimuli [1–3]. An essential response of acinar cells to muscarinic receptor activation is an increase in Ca^{2+} mobilization [4–6]. The resulting elevated cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) activates a number of cellular events, including K^+ efflux via basolateral K^+ channels [7–9], Cl^- efflux via apical anion channels [5,9–11] and Na^+ influx via a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and a Na^+/H^+ exchanger [12–15]. It is now well established that activation of these receptors stimulates hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, producing inositol 1,4,5-trisphosphate (IP_3) and other inositol phosphates [16], which induce a biphasic increase in $[\text{Ca}^{2+}]_i$, an initial rise primarily due to Ca^{2+} release from intracellular stores and a sustained rise as a result of Ca^{2+} influx from the extracellular environment [17–19].

The IP_3 -sensitive Ca^{2+} store, also termed the rapidly exchanging Ca^{2+} store (RECS), is probably localized in the rough endoplasmic reticulum and releases Ca^{2+} through channels that are IP_3 receptors [16,20–22]. The RECS has been widely studied and well characterized in many cell types [23]. Recent studies indicate, however, that more than one intracellular Ca^{2+} store is present in many cells. For instance, most cells possess one or more slowly exchanging Ca^{2+} stores (SECS), which equilibrate slowly and are not sensitive to IP_3 or to the Ca^{2+} -ATPase inhibitor thapsigargin (TG) [23]. Accumulation of $^{45}\text{Ca}^{2+}$ in these Ca^{2+} stores is slow and takes several hours to days to reach

isotopic equilibrium [23]. Multiple intracellular Ca^{2+} stores have been identified in neurons [24–26], lymphocytes [27], pancreatic duct epithelial cells [28] and fibroblasts [29]. Little is known, however, about the function, location and regulation of these stores.

It is now well established that an IP_3 -sensitive RECS is present in rat submandibular acinar cells [10,14,30,31], but it is not known if other Ca^{2+} stores are present in these cells. Since Ca^{2+} stores are crucial in controlling cytosolic Ca^{2+} , which plays a critical role in regulating fluid and electrolyte secretion in salivary cells, it is important to elucidate whether IP_3 -insensitive Ca^{2+} stores are also present in salivary acinar cells. In the present study, we examined therefore the Ca^{2+} stores present in rat submandibular acinar cells. The results indicate that these cells have an IP_3 -sensitive RECS, a partially overlapping but larger Ca^{2+} store sensitive to ionomycin and an acidic IP_3 -insensitive Ca^{2+} store which may be a SECS associated with secretory granules.

MATERIALS AND METHODS

Materials

Collagenase (type CLSPA) was from Worthington Biomedical (Malvern, PA, U.S.A.). Acetylcholine (ACh), BSA (type V), EGTA, gramicidin D, Hepes, hyaluronidase (type V), IP_3 , ionomycin, isoprenaline, monensin, noradrenaline and TG were

Abbreviations used: ACh, acetylcholine; BME, basal medium Eagle amino acids; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; IP_3 , inositol 1,4,5-trisphosphate; KRH, Krebs–Hepes physiological salt solution; RECS, rapidly exchanging Ca^{2+} store; SECS, slowly exchanging Ca^{2+} store; TG, thapsigargin.

from Sigma (St. Louis, MO, U.S.A.). Basal medium Eagle amino acids (BME) was from Gibco (Grand Island, NY, U.S.A.). $^{45}\text{CaCl}_2$ was from NEN (Boston, MA, U.S.A.). Fura 2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals used were of the highest grade available.

Solutions

Digestion medium consisted of (in mM): 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 units of collagenase/mg of wet tissue and 10 units of hyaluronidase/mg of wet tissue. pH was adjusted to 7.4 with NaOH after gassing with O_2 at 37 °C for 45 min. Krebs–Hepes solution (KRH) contained (in mM): 120 NaCl, 4 KCl, 15 Hepes, 10 glucose, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 1.0 CaCl_2 , 1% (v/v) BME and 0.01% (w/v) BSA. pH was adjusted to 7.4 after gassing with O_2 for 45 min. Determination of cytosolic free Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ was carried out in a Ca^{2+} -free KRH solution that contained the same components as KRH except that Ca^{2+} was omitted and 0.1 μM EGTA was added. In Na^+ -free solutions, NaCl was replaced by choline chloride. $^{45}\text{Ca}^{2+}$ uptake and release were measured in saponin-permeabilized cells suspended in a cytosolic-like medium containing (in mM): 100 KCl, 20 NaCl, 15 glucose, 20 Hepes, 5 MgCl_2 , 0.02 EGTA (final free Ca^{2+} 100 nM), 3 ATP, 10 phosphocreatine, 10 units/ml creatine kinase and 0.2% (w/v) BSA. pH was adjusted to 7.2.

Animals

Male 150–200 g Sprague–Dawley rats (Sasco Laboratories, Omaha, NB, U.S.A.) were used in all experiments. Rats were kept in individual cages in a room with a controlled photoperiod (12 h/12 h light/dark cycle) and temperature (23 °C) and with standard rat chow and water available *ad libitum*.

Isorenaline treatment was performed by intraperitoneal injection of 20 mg/kg body weight as previously described [32,33]. After 2 h submandibular acini were isolated and Ca^{2+} mobilization was measured (see below).

Preparation of dispersed submandibular acini

Rat submandibular acini were isolated as previously described [5,14]. Briefly, the submandibular glands were rapidly minced and incubated in an oxygenated dispersion medium containing 3 units of collagenase/mg of wet tissue weight and 10 units of hyaluronidase/mg of wet tissue weight at 37 °C in a shaking water-bath for 60 min. After digestion, the preparation was centrifuged at 50 *g* for 2 min and the supernatant was discarded. The preparation was then resuspended in 10 ml of oxygenated washing medium, passed through a four-layer gauze, washed twice by centrifugation at 50 *g* for 2 min, and finally resuspended in fresh medium and kept at room temperature.

Determination of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was determined using the Ca^{2+} -sensitive fluorescent indicator fura 2 as previously described [10,14]. Isolated submandibular acini were loaded with fura 2 by incubation with 1 μM fura 2 acetoxymethyl ester for 30 min at room temperature (23 °C). $[\text{Ca}^{2+}]_i$ was measured using 2 ml of fura-2-loaded acini suspended in Ca^{2+} -free medium in a 4 ml cuvette and fluorescence monitored with a PTI Deltascan 2.060a fluorimeter (PTI Inc., S. Brunswick, NJ, U.S.A.). The excitation wavelengths used were 340 and 380 nm and emission wavelength was 505 nm. Calibration of $[\text{Ca}^{2+}]_i$ was performed for each measurement trace as previously described [10,14]. Fluorescence ratios of the

340/380 nm excitation and 505 nm emission were converted into $[\text{Ca}^{2+}]_i$ as described by Grynkiewicz et al. [34] using 224 nM as the K_d of fura 2 for Ca^{2+} .

Measurement of $^{45}\text{Ca}^{2+}$ release in permeabilized cells

$^{45}\text{Ca}^{2+}$ release was determined as previously described [31]. Isolated submandibular acini were permeabilized by incubation in a cytosolic-like medium containing 45 $\mu\text{g}/\text{ml}$ saponin at 37 °C for 10 min. The acini were then pelleted by centrifugation at 50 *g* for 30 s and resuspended in the same medium without saponin. An ATP-generation system consisting of 6 mM phosphocreatine and 8 units/ml creatine kinase was used to maintain ATP concentration in the reaction system [35]. The reaction mixture also contained the mitochondrial inhibitors oligomycin (10 μM) and antimycin (10 μM) [31]. $^{45}\text{CaCl}_2$ (1 $\mu\text{Ci}/\text{ml}$) was added to the permeabilized acinar suspension and the mixture was incubated at 37 °C for 2 min. The ATP-dependent $^{45}\text{Ca}^{2+}$ uptake was initiated by the addition of 3 mM ATP. After the $^{45}\text{Ca}^{2+}$ content had reached a steady state (in about 30 min), $^{45}\text{Ca}^{2+}$ release was determined by the addition of Ca^{2+} -releasing agents. $^{45}\text{Ca}^{2+}$ retained in the intracellular stores was determined by a rapid filtration technique as previously described [31]. Briefly, a 0.2 ml aliquot of acini was rapidly removed and applied to a 3 μm filter under suction by a suction pump and the filter was washed with 10 ml of ice-cold cytosolic-like medium. The $^{45}\text{Ca}^{2+}$ content was measured by scintillation counting (LS3801, Beckman Instruments, Irvine, CA, U.S.A.).

Data presentation and statistics

All results are presented as means $\pm$ S.E.M. for at least four determinations using different cell preparations. Comparisons were made using the unpaired Student's *t* test. *P* values < 0.05 are considered significant.

RESULTS

IP_3 - and TG-sensitive Ca^{2+} mobilization

Stimulation of isolated submandibular acini with the muscarinic agonist ACh (1 μM) in a Ca^{2+} -free medium induced a rapid transient increase in $[\text{Ca}^{2+}]_i$ (net increase $110 \pm 9\%$; from 90 ± 3 to 188 ± 8 nM; $n = 36$), consistent with previously reported observations [31]. The specific endoplasmic Ca^{2+} -ATPase inhibitor TG (3 μM) induced a 125% increase in $[\text{Ca}^{2+}]_i$ (from 99 ± 3 to 224 ± 10 nM; $n = 14$), which is slightly (16%) larger than the ACh-stimulated Ca^{2+} release ($P > 0.05$). In most cell types, the TG-induced Ca^{2+} release appears to be from the same intracellular site as the IP_3 -sensitive Ca^{2+} release [24,26,36–38]. In some cell types, however, such as Jurkat T-lymphocytes [27] and PANC-1 cells [28], the TG-sensitive store is larger than the IP_3 -sensitive store, and the IP_3 -sensitive store can be completely released by TG. To test whether TG and IP_3 empty the same Ca^{2+} store in submandibular acinar cells, the cells were stimulated with ACh and TG in alternating sequences. After ACh stimulation (1 μM) which induced a $105 \pm 10\%$ ($n = 5$) increase in $[\text{Ca}^{2+}]_i$, TG stimulation resulted in a small $[\text{Ca}^{2+}]_i$ increase ($19 \pm 4\%$, $n = 5$). After administration of TG, which induced a $124 \pm 16\%$ ($n = 6$) increase in $[\text{Ca}^{2+}]_i$, ACh (1 μM) elicited little further increase. These results suggest that ACh and TG induce Ca^{2+} release from the same store, the Ca^{2+} -ATPase-dependent IP_3 -sensitive Ca^{2+} store, but that, as in other cells, the TG-sensitive store is larger than the agonist (and IP_3)-sensitive store.

Figure 1 Ca²⁺ release induced by ionomycin before or after the addition of ACh or TG

[Ca²⁺]_i in fura-2-loaded rat submandibular acini was monitored as described in the Materials and methods section. Acini were treated with 1 μ M ACh and 1 μ M ionomycin (Iono) or 3 μ M TG and 1 μ M ionomycin in different sequences at the time indicated by the arrows. Each trace is a representative response from different cell preparations (in **A**, $n = 13$; in **B**, $n = 8$; in **C**, $n = 6$; in **D**, $n = 6$).

Ionomycin-induced Ca²⁺ mobilization

After stimulation with ACh, addition of the Ca²⁺ ionophore ionomycin (1 μ M) induced a further ($41 \pm 6\%$) increase in [Ca²⁺]_i ($n = 13$; Figure 1A). In a similar fashion, ionomycin induced a $57 \pm 8\%$ further increase in [Ca²⁺]_i after TG treatment ($n = 8$; Figure 1B), suggesting that there is additional stored Ca²⁺ which can be released by ionomycin after the IP₃-sensitive store is depleted or no longer responsive to agonist or IP₃. To test whether ionomycin was able, by itself, to discharge the IP₃-sensitive Ca²⁺ store, cells were first exposed to ionomycin and then either ACh (1 μ M) or TG (3 μ M). As shown in Figures 1(C) and 1(D), ionomycin evoked a $170 \pm 14\%$ net increase in [Ca²⁺]_i (from 110 ± 8 to 299 ± 29 nM, $n = 6$). Subsequent exposure to ACh or TG did not induce any further Ca²⁺ increase (Figures 1C and 1D), indicating that ionomycin completely emptied the IP₃-sensitive store.

Monensin-induced Ca²⁺ mobilization

After the IP₃- and the ionomycin-sensitive stores were presumably emptied, exposing cells to the Na⁺/H⁺ ionophore monensin (10 μ M), which collapses the pH gradient across the limiting membrane of subcellular organelles, elicited a further increase in [Ca²⁺]_i (net increase $267 \pm 49\%$, from 85 ± 4 to 302 ± 35 nM, $n = 9$; Figure 2A). The magnitude of this response was much larger than those seen with ACh, TG or ionomycin alone. Exposure of cells to monensin first induced a $25 \pm 3\%$ increase in [Ca²⁺]_i ($n = 5$) and a subsequent stimulation with ACh (1 μ M) caused a $114 \pm 31\%$ further increase in [Ca²⁺]_i. [Ca²⁺]_i returned to the resting level in less than 3 min both in the absence and presence of the ionophore, suggesting that Ca²⁺ extrusion is not significantly altered by monensin. The monensin-induced Ca²⁺ release was not due to residual Ca²⁺ retained in the IP₃-sensitive store, because, after stimulation with a higher concentration (10 μ M) of ACh, which should empty the IP₃-sensitive store more thoroughly, the ionomycin + monensin-induced increase in

Figure 2 Ca²⁺ release induced by sequential treatment with ACh or TG, ionomycin and monensin

[Ca²⁺]_i in fura-2-loaded submandibular acini was monitored as described in Figure 1. Acini were sequentially exposed to 1 or 10 μ M ACh, 1 μ M ionomycin (Iono) and 10 μ M monensin (Mon) (**A**) or 3 μ M TG, 1 μ M ionomycin and 10 μ M monensin (**B**) at the time indicated by the arrows. Each trace is a representative response using different cell preparations (in **A**, 1 μ M ACh, $n = 9$; 10 μ M ACh, $n = 6$; in **B**, $n = 9$).

[Ca²⁺]_i ($300 \pm 34\%$, $n = 6$; Figure 2A) was comparable with that seen with these drugs after the lower (1 μ M) ACh concentration. A similar increase in [Ca²⁺]_i ($298 \pm 41\%$, $n = 9$) was also observed when monensin was added after TG + ionomycin (Figure 2B).

To characterize the monensin-sensitive store further, the dependence of the monensin-induced Ca²⁺ release on the filling status of the IP₃-sensitive and ionomycin-sensitive stores was examined. As shown in Figure 3, monensin still caused an additional increase in [Ca²⁺]_i after the IP₃-sensitive store was depleted with either ACh or TG, but this increase was significantly less than that observed when monensin was added after ionomycin. After ACh stimulation, monensin resulted in a $46 \pm 15\%$ increase in [Ca²⁺]_i ($n = 8$; Figure 3A). A similar result was observed when TG was followed by monensin ($48 \pm 18\%$, $n = 6$; Figure 3B). A comparison of these observations with those shown in Figure 2 suggests that the large monensin-induced Ca²⁺ release requires that ionomycin be present in the incubation solution and that the Ca²⁺ release induced by ionomycin + monensin is from a Ca²⁺ store separate from that discharged by ACh, TG or ionomycin alone.

Ionomycin-dependence of the monensin-induced Ca²⁺ release

The clearly important role of ionomycin in the monensin-induced Ca²⁺ release was then examined further. Exposure to monensin alone caused a $67 \pm 9\%$ increase in [Ca²⁺]_i ($n = 5$; Figure 4A, +Na⁺). However, when added after monensin, ionomycin induced a large Ca²⁺ release ($290 \pm 32\%$, $n = 5$; Figure 4A, +Na⁺). Because ionomycin itself only induced a 150–170% increase in [Ca²⁺]_i (see Figure 1), the significantly larger Ca²⁺ release after this ionophore shown in Figure 4 confirms that the

Figure 3 Ca^{2+} release induced by monensin after ACh or TG

$[\text{Ca}^{2+}]_i$ in fura-2-loaded submandibular acini was monitored as described in Figure 1. Acini were sequentially exposed to $1 \mu\text{M}$ ACh and $10 \mu\text{M}$ monensin (Mon) (A) or to $3 \mu\text{M}$ TG and $10 \mu\text{M}$ monensin (B) at the time indicated by the arrows. Each trace is a representative response using different cell preparations (in A, $n = 8$; in B, $n = 6$).

monensin-induced Ca^{2+} release after ACh+ionomycin or TG+ionomycin (Figure 2) is ionomycin-dependent. This hypothesis was tested by exposing acini to ionomycin first, followed by monensin without a previous ACh or TG stimulus. Compared with the results shown in Figure 2, Figure 4(B) (+ Na^+) shows that ionomycin induced a $163 \pm 32\%$ increase in $[\text{Ca}^{2+}]_i$ ($n = 4$), and that the subsequent administration of monensin caused a further $322 \pm 27\%$ increase (Figure 4B, + Na^+). These results indicate that Ca^{2+} release from the Ca^{2+} store that is apparently sensitive to monensin requires co-exposure to ionomycin and monensin. Neither of the ionophores alone elicited substantial Ca^{2+} release from this store.

Role of Na^+ in the ionomycin- and monensin + ionomycin-induced Ca^{2+} mobilization

Since monensin induces an increase in cytosolic Na^+ content in rat submandibular acinar cells [14], the possible role of $\text{Na}^+/\text{Ca}^{2+}$ exchange in the Ca^{2+} mobilization observed in the presence of monensin was examined. As shown in Figure 4(A) (– Na^+), in the absence of Na^+ , the monensin- and the monensin + ionomycin-induced Ca^{2+} release was markedly reduced. Exposure to $10 \mu\text{M}$ monensin in Na^+ -free medium induced a $25 \pm 3\%$ ($n = 4$) increase in $[\text{Ca}^{2+}]_i$ (Figure 4A, – Na^+) which was significantly smaller than that in Na^+ -containing medium ($P < 0.01$). Subsequent exposure to ionomycin ($1 \mu\text{M}$) only caused a $102 \pm 20\%$ ($n = 4$) further increase in $[\text{Ca}^{2+}]_i$, which was also significantly smaller than that in Na^+ -containing medium ($P < 0.005$; Figure 4A, – Na^+). On the other hand, exposure to ionomycin first in a Na^+ -free medium elicited an $83 \pm 11\%$ increase in $[\text{Ca}^{2+}]_i$ ($n = 4$), which was 50% (but not statistically significantly) smaller than in Na^+ -containing medium ($P > 0.05$). A subsequent addition of monensin induced a $128 \pm 20\%$ ($n = 4$)

Figure 4 Ca^{2+} release induced by ionomycin and monensin in the absence and presence of external Na^+

$[\text{Ca}^{2+}]_i$ in fura-2-loaded submandibular acini was monitored as described in Figure 1. (A) Acini were sequentially exposed to $10 \mu\text{M}$ monensin (Mon) and $1 \mu\text{M}$ ionomycin (Iono) in the absence (– Na^+) and presence (+ Na^+) of external Na^+ . (B) Acini were sequentially exposed to ionomycin and monensin in the absence (– Na^+) and presence (+ Na^+) of external Na^+ . Ionomycin and monensin were added at the time indicated by the arrows. Each trace is the representative response using different cell preparations (in A: + Na^+ , $n = 5$; – Na^+ , $n = 4$; in B: + Na^+ , $n = 5$; – Na^+ , $n = 4$).

further elevation in $[\text{Ca}^{2+}]_i$, which was significantly smaller than that in Na^+ -containing medium ($P < 0.001$; Figure 4B). These results suggest two possibilities: first, a $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism may mediate the monensin- and monensin + ionomycin-induced Ca^{2+} release; and second, Na^+ is required to collapse the pH gradient present in the intracellular organelle associated with this Ca^{2+} store. To investigate these possibilities, the Na^+ ionophore gramicidin was used to increase cytosolic Na^+ concentration. It is well established that gramicidin effectively increases cytosolic Na^+ concentrations in several minutes in most cell types, including salivary acinar cells [39]. Exposure of rat submandibular acinar cells to gramicidin ($5 \mu\text{M}$) caused a $30 \pm 3\%$ ($n = 4$) increase in $[\text{Ca}^{2+}]_i$, and subsequent ionomycin treatment induced a $114 \pm 4\%$ ($n = 4$) further increase in $[\text{Ca}^{2+}]_i$ (Figure 5A). Gramicidin did not significantly change Ca^{2+} removal from the cytoplasmic space. $[\text{Ca}^{2+}]_i$ returned to the prestimulation level in less than 3 min after stimulation with ACh ($1 \mu\text{M}$) in the presence of gramicidin. The gramicidin + ionomycin-induced increase in $[\text{Ca}^{2+}]_i$ was significantly smaller than the monensin + ionomycin-induced increase ($P < 0.005$; Figure 5A). Exposure to gramicidin after ionomycin induced a $20 \pm 6\%$ ($n = 6$) further increase in $[\text{Ca}^{2+}]_i$, which was significantly smaller than that induced by monensin after ionomycin ($P < 0.001$; Figure 5B). These results suggest that an elevation of cytosolic Na^+ concentration does not, by itself, cause significant Ca^{2+} release and that this manoeuvre does not result in a large release of Ca^{2+} by ionomycin. These data suggest that the monensin + ionomycin-induced Ca^{2+} release is not primarily mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange. However, an increase in

Figure 5 Effects of monensin and gramicidin on ionomycin-induced Ca²⁺ release

Fura-2-loaded rat submandibular acini were sequentially exposed to 10 μ M monensin (Mon) or 5 μ M gramicidin (Gram) and 1 μ M ionomycin (Iono) and [Ca²⁺]_i was monitored. (A) Acini were exposed to either monensin or gramicidin at the time indicated by the first arrow, followed by 1 μ M ionomycin (Mon, $n = 5$; Gram, $n = 4$). (B) Acini were exposed to 1 μ M ionomycin followed by either 5 μ M gramicidin or 10 μ M monensin at the time indicated by the second arrow (Mon, $n = 5$; Gram, $n = 6$).

cytosolic Na⁺ may be required to collapse the pH gradient by monensin since transmembrane Na⁺ movement facilitated by monensin is counter-balanced by H⁺ movement in the opposite direction [40].

Localization of the monensin + ionomycin-sensitive Ca²⁺ store

Since exposure to ionomycin + monensin induced a much larger release of Ca²⁺ than that seen from the IP₃- or TG-sensitive store, it is clear that the capacity of the latter store is considerably larger than that of the agonist-sensitive one. Because Ca²⁺ release is elicited after exposure to monensin, this store is acidic. Two subcellular organelles are the most likely location of this acidic IP₃-insensitive store, mitochondria and secretory granules. To explore these possibilities further, the release of ⁴⁵Ca²⁺ from tracer-preloaded cells was investigated. As shown in Figure 6 and Table 1, the ⁴⁵Ca²⁺ release induced by IP₃ and TG were identical in the absence and presence of mitochondrial inhibitors (about 35 %, Table 1), but the ionomycin-induced ⁴⁵Ca²⁺ release in the absence of mitochondrial inhibitors was slightly greater than that seen in the presence of the inhibitors (Table 1). The monensin-induced ⁴⁵Ca²⁺ release, on the other hand, was quite small (3–10 %, Table 1), and even less in the absence of mitochondrial inhibitors (Table 1). It is interesting to note that the monensin-induced ⁴⁵Ca²⁺ release was very small in both the absence and presence of mitochondrial inhibitors (Figure 6 and Table 1), and that IP₃ (or TG) and ionomycin discharged most of the accumulated ⁴⁵Ca²⁺. To confirm this observation, ⁴⁵Ca²⁺-loaded cells were stimulated by 5 μ M IP₃ and 3 μ M TG followed by 10 μ M monensin. IP₃ and TG induced a 52 % release of

Figure 6 ⁴⁵Ca²⁺ release from permeabilized submandibular acini

Isolated rat submandibular acini were permeabilized with 45 μ g/ml saponin for 10 min in a cytosolic-like medium and loaded with 1 μ Ci/ml ⁴⁵CaCl₂ in the cytosolic-like medium containing an ATP-regeneration system in the absence (○) or presence (□) of mitochondrial inhibitors. ⁴⁵Ca²⁺ release was activated by sequential addition of 5 μ M IP₃ or 3 μ M TG followed by 1 μ M ionomycin (Iono) and 10 μ M monensin (Mon) at the time indicated by the arrows.

Table 1 ⁴⁵Ca²⁺ release from the saponin-permeabilized submandibular cells

Isolated rat submandibular acini were permeabilized with 45 μ g/ml saponin for 10 min in a cytosolic-like medium and loaded with 1 μ Ci/ml ⁴⁵CaCl₂ in the same medium containing an ATP-regeneration system in the absence or presence of mitochondrial inhibitors. ⁴⁵Ca²⁺ release was activated by sequential (5 min intervals) addition of 5 μ M IP₃ or 3 μ M TG followed by 1 μ M ionomycin and 10 μ M monensin. Values are means $\pm$ S.E.M. for the number of experiments shown in parentheses.

Treatment	⁴⁵ Ca ²⁺ release (%)	
	— Inhibitors	+ Inhibitors
IP ₃ (5 μ M)	34.0 $\pm$ 4.3 (5)	34.1 $\pm$ 3.6 (7)
TG (3 μ M)	36.0 $\pm$ 3.5 (4)	33.2 $\pm$ 2.7 (5)
Ionomycin (1 μ M)		
After IP ₃	56.2 $\pm$ 6.7 (5)	49.8 $\pm$ 8.2 (4)
After TG	57.0 $\pm$ 7.3 (4)	46.3 $\pm$ 7.1 (4)
Monensin (10 μ M)		
After IP ₃ + Ionomycin	3.8 $\pm$ 1.1 (5)	7.0 $\pm$ 2.1 (4)
After TG + Ionomycin	3.3 $\pm$ 0.9 (4)	10.3 $\pm$ 3.9 (4)

⁴⁵Ca²⁺ and monensin induced a further 10 % ⁴⁵Ca²⁺ release ($n = 4$). This result indicates that monensin induces only a small release, 50 % of loaded ⁴⁵Ca²⁺ is still being undischarged. The simplest explanation of the discrepancy seen between the monensin effects in the fura 2 experiments with intact cells (Figure 2) and the ⁴⁵Ca²⁺ experiments in permeabilized cells (Figure 6) is that the radiotracer was not taken up by the store that is discharged by ionomycin + monensin. This could occur if Ca²⁺ uptake into this store is either too slow to measure in short-term experiments like ours or is not dependent on an ATP-energized Ca²⁺-ATPase like that present in the IP₃- and ionomycin-sensitive stores.

Secretory granules generally represent an acidic compartment

Figure 7 Effect of degranulation on Ca^{2+} release

Fura-2-loaded acini were sequentially exposed to $10 \mu M$ ACh, $1 \mu M$ ionomycin (Iono) and $10 \mu M$ monensin (Mon) at the time indicated by the arrows. (A) $[Ca^{2+}]_i$ response in acini prestimulated with $10 \mu M$ isoprenaline and $10 \mu M$ noradrenaline for 45 min (Treated). After treatment, acini were rinsed with KRH containing 0.01% BSA and resuspended in a Ca^{2+} -free KRH and exposed to ACh, Iono and Mon. Control cells (Control) were treated with vehicle only. (B) $[Ca^{2+}]_i$ response in acini isolated from rats treated *in vivo* with isoprenaline. Rats were injected intraperitoneally with 20 mg/kg body weight isoprenaline and 2 hours later, the submandibular glands were removed and acinar cells were isolated. Each trace is a representative response from separate experiments (in A: control, $n = 9$; treated, $n = 8$; in B: $n = 5$).

in various cells [41]. The possibility that secretory granules may be the source of the Ca^{2+} released by ionomycin + monensin in submandibular cells was tested by inducing granule discharge with the β -adrenergic agonist isoprenaline and the α_1 -adrenergic agonist noradrenaline. It has been previously shown that combined stimulation of both cAMP-dependent and Ca^{2+} -dependent pathways greatly augments the rate of exocytosis [42] and that stimulation of rat submandibular cells with isoprenaline plus noradrenaline discharges most secretory granules by exocytosis [32,42]. In the present study, cells were exposed to $10 \mu M$ noradrenaline + $10 \mu M$ isoprenaline for 45 min and then to the sequence of drugs shown in Figure 2. As shown in Figure 7(A), this treatment abolished the ACh-induced increase in $[Ca^{2+}]_i$. Obviously, this is due to α -adrenergic receptor-stimulation-induced Ca^{2+} release from the IP_3 -sensitive stores. After exposure to ACh and ionomycin, exposure to monensin caused a $35 \pm 4\%$ increase in $[Ca^{2+}]_i$ (from 114 ± 13 to 153 ± 17 nM, $n = 8$; Figure 7A). This effect was significantly lower than that seen in cells not exposed to isoprenaline + noradrenaline (Figure 7A) and represented an 87% decrease in Ca^{2+} release ($P < 0.001$). These results suggest that the Ca^{2+} released by ionomycin + monensin is probably associated with secretory granules.

To confirm this observation, an *in vivo* degranulation experiment was conducted by injecting rats with 20 mg/kg body weight isoprenaline. This treatment has been well documented to completely degranulate the submandibular acinar cells [32,33].

Cells were isolated 2 h after the injection and exposed to agonist, ionomycin and monensin in sequence (Figure 7B). The ionomycin + monensin-induced Ca^{2+} release in acini isolated from these isoprenaline-treated rats was dramatically reduced ($69 \pm 9\%$, $n = 5$; $P < 0.001$; Figure 7B) when compared with that seen in control acini, which supports the view that ionomycin + monensin-induced Ca^{2+} release is from secretory granules.

DISCUSSION

This study provides evidence that multiple Ca^{2+} stores are present in rat submandibular acinar cells. At least three Ca^{2+} stores were distinguishable under the conditions of this study. First, the ACh-induced transient increase in $[Ca^{2+}]_i$ is from the IP_3 -sensitive Ca^{2+} store, a well-characterized store also referred to as RECS. As discussed elsewhere [23,43], the RECs is believed to be located in the endoplasmic reticulum. However, recent studies suggest that the early IP_3 -sensitive Ca^{2+} release may also be from the nuclear envelope [44] or the secretory granules [45,46] in pancreatic acinar cells. Obviously, the location of the RECS may not be limited to the endoplasmic reticulum. The physiological significance of the heterogeneous location of the RECS may be the rapid activation of the local ion transport mechanisms such as ion channels located in the same area [47].

Second, an IP_3 -insensitive but ionomycin-sensitive Ca^{2+} release was identified in this study. After Ca^{2+} release was induced by ACh or TG, ionomycin elicited a further release. The location of the ionomycin-sensitive Ca^{2+} store(s) is still unclear. Ionomycin was shown to discharge the agonist- (and TG)-sensitive store completely when applied before these drugs, which indicates a degree of overlap in the distribution of IP_3 (and TG)- and ionomycin-sensitive Ca^{2+} release. Release from the ionomycin-sensitive store, however, does not appear to be through IP_3 -sensitive channels, since IP_3 discharged only a fraction of the $^{45}Ca^{2+}$ in permeabilized cells, whereas ionomycin discharged a larger fraction of the radiotracer. In these cells, the ionomycin-induced $^{45}Ca^{2+}$ release was the largest portion of Ca^{2+} release, but mitochondrial inhibitors only suppressed a small part ($\sim 10\%$) of this ionomycin-induced $^{45}Ca^{2+}$ release, suggesting that only a small part of the IP_3 -insensitive and ionomycin-sensitive Ca^{2+} release is from mitochondria. These observations suggest that ionomycin possibility affects both IP_3 -sensitive and -insensitive portions of a larger and more heterogeneous Ca^{2+} store. Our results show a discrepancy in the extent of the Ca^{2+} -release response to ionomycin in the fura 2 and $^{45}Ca^{2+}$ experiments. The reasons for this difference are not clear at present. Other cells have been shown to have IP_3 -insensitive Ca^{2+} stores which can be discharged by a variety of substances [23], but our current results do not allow us to identify whether the ionomycin-sensitive store shown in this study corresponds to any of these other stores. This issue is currently under further investigation.

Third, our studies also indicate that a large and acidic IP_3 -insensitive Ca^{2+} store is present in submandibular acinar cells. After Ca^{2+} was released from the IP_3 -sensitive and the IP_3 -insensitive ionomycin-sensitive stores, monensin triggered a further 3-fold increase in $[Ca^{2+}]_i$ in the continued presence of ionomycin. This Ca^{2+} release was not mediated by Na^+/Ca^{2+} exchange, since an increase in cytosolic Na^+ produced by gramicidin without collapsing the pH gradient induced a significantly smaller Ca^{2+} release than that seen with monensin. This store is obviously acidic, because ionomycin by itself did not induce Ca^{2+} release from it and a collapse of the pH gradient was required for significant Ca^{2+} release. It has been well established

that ionomycin is unable to discharge acidic Ca²⁺ stores unless the pH gradient is collapsed by NH₄Cl or monensin [48]. The experiments shown in Figures 2 and 4 clearly demonstrate that both ionomycin and monensin were required to discharge this store. Monensin or ionomycin alone only induced a small Ca²⁺ release, but the combination of the two reagents evoked a significantly larger release, regardless of the sequence of treatment. In contrast with the observations with fura-2-loaded intact cells, ⁴⁵Ca²⁺ release from permeabilized and tracer-preloaded cells showed that only up to 10% of preloaded ⁴⁵Ca²⁺ was released by monensin after ionomycin. This suggests that the radiotracer was not loaded into this store and that Ca²⁺ uptake into this store was either too slow or not dependent on Ca²⁺-ATPase.

Our results provide some evidence for the location of the IP₃-insensitive and monensin + ionomycin-sensitive Ca²⁺ store. Results in Figure 7 clearly suggest that this Ca²⁺ store is associated with the secretory granules. First, mitochondria are not the location of the SECS, since the monensin-induced ⁴⁵Ca²⁺ release from cells preloaded with ⁴⁵Ca²⁺ in the absence and presence of mitochondrial inhibitors were comparable. Second, discharge of secretory granules with isoprenaline significantly reduced the monensin + ionomycin-induced Ca²⁺ release. The capacity of this store seems to be larger than that of the IP₃-sensitive and IP₃-insensitive but ionomycin-sensitive stores, since the [Ca²⁺]_i increase caused by the combination of ionomycin and monensin (300%) was larger than that caused by ACh or TG (126%) or by ionomycin alone (50%). Morphologically, a large portion of the cellular space is filled with secretory granules in salivary gland cells. For example, about 30 and 55–59% of the cell volume is occupied by secretory granules in the acinar cells of parotid [49] and submandibular gland [50] respectively. Therefore secretory granules represent a potentially large repository for significant Ca²⁺ storage. Indeed, the total Ca²⁺ content of secretory granules determined by electron X-ray microanalysis is much higher than that of other subcellular organelles in all secretory cells studied [41] including parotid [51] and submandibular acinar cells [52]. Most secretory granules contain millimolar or higher concentrations (~200 mM) of total Ca²⁺, which is several orders of magnitude higher than [Ca²⁺]_i [41]. The granular store is likely to be of the slowly exchanging variety of Ca²⁺ stores, which equilibrate slowly and show IP₃-insensitivity [23]. This would be consistent with previous observations showing that the Ca²⁺ in the secretory granules was not readily exchangeable with extragranular Ca²⁺ in parotid cells [53].

The physiological significance of the IP₃-insensitive pool apparently associated with secretory granules is still uncertain. Granular Ca²⁺ may regulate exocytosis in β -cells and sea-urchin eggs [41] and protein secretion in salivary cells [53]. Its possible involvement in the regulation of free cytosolic Ca²⁺ has been suggested [41,45,46], but is not universally accepted since Ca²⁺ release from secretory granules has not been observed under physiological conditions. The granular store of rat submandibular cells apparently lacks IP₃ receptors and may not be involved in the release of Ca²⁺ induced by ACh and other IP₃-generating agents.

In summary, the present study indicates the following (1) Rat submandibular acinar cells have three separate intracellular Ca²⁺ stores, including an IP₃-sensitive one, an IP₃-insensitive but ionomycin-sensitive one and an IP₃-insensitive but ionomycin + monensin-sensitive one. (2) The ionomycin-sensitive store includes the IP₃- and TG-sensitive stores and other cellular compartments that lack IP₃ receptors or TG-sensitive Ca²⁺ pumps. A small portion is apparently associated with mitochondria. (3) The ionomycin + monensin-sensitive Ca²⁺ store is

a large acidic pool associated with secretory granules, which releases Ca²⁺ only when the pH gradient across the limiting membrane is collapsed. Ca²⁺ uptake into this pool is slow and not driven by a Ca²⁺-ATPase mechanism. Future studies should be directed to two important issues: the mechanisms mediating Ca²⁺ uptake and release from this store under physiological conditions and the physiological significance of this large SECS.

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REFERENCES

- Baum, B. J. (1987) *J. Dent. Res.* **66**, 628–632
- Martinez, J. R. (1987) *J. Dent. Res.* **66**, 638–647
- Yu, J. H. (1990) *Arch. Oral Biol.* **35**, 639–643
- Cook, D. I., Van Lennep, E. W., Roberts, M. L. and Young, J. A. (1994) in *Physiology of the Gastrointestinal Tract*, 3rd edn. (Johnson, L. R., ed.), pp. 1061–1117, Raven Press, New York
- Martinez, J. R. and Cassity, N. (1986) *Pflugers Arch.* **407**, 615–619
- Putney, Jr., J. W. (1986) *Annu. Rev. Physiol.* **48**, 75–88
- Martinez, J. R. and Camden, J. (1982) *Arch. Oral Biol.* **27**, 939–944
- Martinez, J. R. and Quissell, D. O. (1976) *J. Pharmacol. Exp. Ther.* **199**, 518–525
- Petersen, O. H. (1992) *J. Physiol. (London)* **448**, 1–51
- Martinez, J. R. and Camden, J. (1989) *Arch. Oral Biol.* **34**, 147–152
- Nauntofte, B. and Poulsen, J. H. (1986) *Am. J. Physiol.* **251**, C175–C185
- Dissing, S. and Nauntofte, B. (1990) *Am. J. Physiol.* **259**, G1044–G1055
- Martinez, J. R. and Barker, S. (1988) *Arch. Oral Biol.* **33**, 621–622
- Martinez, J. R., Camden, J. and Barker, S. (1990) *Arch. Oral Biol.* **35**, 359–364
- Zhang, G. H., Cragoe, Jr., E. J. and Melvin, J. E. (1993) *Am. J. Physiol.* **264**, C54–C62
- Berridge, M. J. and Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Melvin, J. E., Koek, L. and Zhang, G. H. (1991) *Am. J. Physiol.* **261**, G1043–G1050
- Merritt, J. E. and Rink, T. J. (1987) *J. Biol. Chem.* **262**, 4958–4960
- Nauntofte, B. and Dissing, S. (1987) *Am. J. Physiol.* **251**, C175–C185
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Berridge, M. J. and Irvine, R. F. (1989) *Nature (London)* **341**, 197–205
- Streb, H., Irvine, R. F., Berridge, M. J. and Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) *Physiol. Rev.* **74**, 595–636
- Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J. and Pozzan, T. (1991) *J. Biol. Chem.* **266**, 20159–20167
- Nori, A., Villa, A., Podini, P., Wither, D. R. and Volpe, P. (1993) *Biochem. J.* **291**, 199–204
- Zacchetti, D., Clementi, E., Fasolato, C., Lorenzon, P., Zottini, M., Grohovaz, F., Fumagalli, G., Pozzan, T. and Meldolesi, J. (1991) *J. Biol. Chem.* **266**, 20152–20158
- Guse, A. H., Roth, E. and Emmrich, F. (1993) *Biochem. J.* **291**, 447–451
- Wu, L., Katz, S. and Brown, G. R. (1994) *Cell Calcium* **15**, 228–240
- Baumgarten, L. B., Lee, H. C. and Villereal, M. L. (1995) *Cell Calcium* **17**, 41–52
- Hurley, T. W. and Brinck, R. W. (1992) *Arch. Oral Biol.* **37**, 763–769
- Seagrave, J. C., Barker, S., Martinez, A. M. and Martinez, J. R. (1993) *Proc. Soc. Exp. Biol. Med.* **203**, 490–500
- Bogart, B. I. (1975) *J. Ultrastruct. Res.* **52**, 139–155
- Simson, J. A. V., Spicer, S. S., Setser, M. E. and Martinez, J. R. (1978) *Lab. Invest.* **39**, 17–166
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. and Putney, Jr., J. W. (1984) *Biochem. J.* **224**, 741–746
- Gamberucci, A., Fulceri, R., Tarroni, P., Giunti, R., Marcolongo, P., Sorrentino, V. and Benedetti, A. (1995) *Cell Calcium* **17**, 431–441
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Zhang, G. H. and Melvin, J. E. (1993) *FEBS Lett.* **327**, 1–6
- Zhang, G. H. and Melvin, J. E. (1996) *J. Biol. Chem.*, in the press
- Cussler, E. L., Evans, D. F. and Matesich, S. M. A. (1971) *Science* **172**, 377–379
- Nicaise, G., Maggio, K., Thirion, S., Horoyan, M. and Keicher, E. (1992) *Biol. Cell* **75**, 89–99
- Quissell, D. O. (1993) in *Biology of the Salivary Glands* (Dobrosielski-Vergona, K., ed.), pp. 181–200, CRC Press, Boca Raton, FL
- Toescu, E. C. (1995) *Am. J. Physiol.* **269**, G173–G185
- Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V. and Petersen, O. H. (1995) *Cell* **80**, 439–444
- Kasai, H., Li, Y. and Miyashita, Y. (1993) *Cell* **74**, 669–677

- 46 Thorn, P., Lawrie, A. M., Smith, P. M., Gallacher, D. V. and Petersen, O. H. (1993) *Cell* **74**, 661–668
- 47 Kasai, H. and Petersen, O. H. (1994) *Trends Neurol. Sci.* **17**, 95–101
- 48 Pressman, B. C. and Fahim, M. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22**, 465–490
- 49 Anderson, L. C., Garrett, J. R., Suleiman, A. H., Proctor, G. B., Chan, K.-M. and Hartley, R. (1993) *Cell Tissue Res.* **274**, 559–566
- 50 Bloom, G. D., Carlsoo, B., Danielsson, A., Gustafsson, H. and Henriksson, R. (1979) *Med. Biol.* **57**, 224–233
- 51 Izutsu, K. and Johnson, D. E. (1986) *J. Physiol. (London)* **381**, 297–309
- 52 Mörk, A.-C., Zhang, A., Martinez, J. R. and Roomans, G. M. (1996) *Histochem. Cell Biol.* **105**, 297–303
- 53 Wallach, D. and Schramm, M. (1971) *Eur. J. Biochem.* **21**, 433–437

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