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Evidence for Reduced Capacity for Ca^{2+} Removal from the Cytosol in Submandibular Cells of Newborn Rats

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The increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) seen in submandibular cells of early postnatal rats following exposure to acetylcholine (ACh) is larger than in cells of adult rats. To elucidate possible reasons for this difference, we compared Ca^{2+} movements through Ca^{2+} pumps in both types of cells using Ca^{2+}-sensitive fluorescent probe fura-2 and the radiotracer $^{45}Ca^{2+}$. Ca^{2+} release induced by endoplasmic reticulum (ER) Ca^{2+}-pump inhibitor thapsigargin (TG) was significantly smaller in neonatal cells than in adult cells, whereas the inositol 1,4,5-trisphosphate (IP_3)-elicited Ca^{2+} release was comparable in both cell types. This suggests that although the size of the IP_3-sensitive Ca^{2+} pool is adequate in immature cells, the activity of TG-sensitive Ca^{2+} pump in this pool is lower. The activity of the plasma membrane (PM) Ca^{2+}-pump, measured by extrusion of $^{45}Ca^{2+}$, was also significantly lower in immature cells. These results indicate that both ER and PM Ca^{2+} pumps may be functionally underdeveloped in immature cells, and that the enhanced increase of $[Ca^{2+}]_i$ seen in response to ACh in immature cells may be partially, if not completely, due to a reduced capacity for removal of Ca^{2+} from the cytosol by active mechanisms.

The inositol phosphate (IP)-Ca^{2+} signaling system of rat submandibular acinar cells undergoes a unique maturation pattern during postnatal development (1-4). The generation of inositol 1,4,5-trisphosphate (IP_3) and the increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to acetylcholine are significantly higher in cells of immature glands than in those of mature glands (2,3). In contrast, the ATP-dependent $^{45}Ca^{2+}$ uptake and the IP_3-induced $^{45}Ca^{2+}$ release in permeabilized cells are lower in cells of immature rats (3). The underlying mechanism mediating this intriguing pattern of IP-Ca^{2+} signaling in cells of immature rats remains unknown. While several possibilities may be account for the enhanced $[Ca^{2+}]_i$ increase seen in fura-2 experiments, a reduced capacity to remove Ca^{2+} from the cytosol is likely to be one of the major reasons. Under normal conditions, $[Ca^{2+}]_i$ is tightly regulated at a low concentration (about 100 nM) and excess Ca^{2+} is removed from the cytosol by two Ca^{2+}-pumps. One is associated with the endoplasmic reticulum (ER), which causes Ca^{2+} reuptake into the Ca^{2+} pool, and another is associated with the plasma membrane (PM), which extrudes Ca^{2+} to the extracellular fluid. These pumps may be underdeveloped in salivary cells of immature animals, which could explain the slower and smaller Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} pool (2,3) and a reduced Ca^{2+} extrusion. Since Ca^{2+} pumps play a central role in Ca^{2+} removal from the cytosol, we compared, therefore, the activity of the ER and PM Ca^{2+} pumps in submandibular acinar cells of newborn and adult rats.

MATERIALS AND METHODS

Solutions. The 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) of BSA, 1% BME (v/v), 3 U/mg wet tissue of collagenase (type CLSPA), 10 U/mg wet tissue of hyaluronidase. pH was adjusted to 7.4 with NaOH after gassing with O_2 at 37°C for 45 min. Krebs-Hepes solution (KRS) 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% BME Amino Acids, 0.01% (w/v) BSA. pH was adjusted to 7.4 after gassing with O_2 for 45 min. Ca^{2+}-free KRS contained

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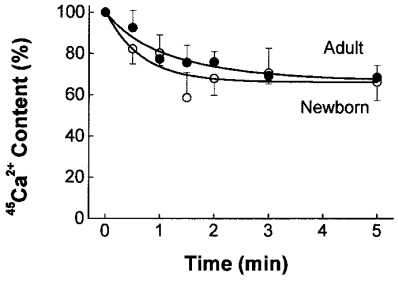

FIG. 1. $^{45}Ca^{2+}$ release induced by inositol 1,4,5-trisphosphate. Isolate submandibular acinar cells from adult (*Adult*) and newborn (*Newborn*) rats were permeabilized with saponin and loaded with the radiotracer $^{45}Ca^{2+}$ for 30 min. After loading, the cells were exposed to 5 μM inositol 1,4,5-trisphosphate (IP_3) at the time point 0 min. Adult, n = 3; newborn, n = 3.

the same component as KRS, except $CaCl_2$ was omitted and the free Ca^{2+} concentration was adjusted to about 100 nM with EGTA. The cytosolic-like medium contained (in mM): 100 KCl, 20 NaCl, 15 glucose, 20 Hepes (pH 7.2), 5 $MgCl_2$, 0.02 EGTA (final free Ca^{2+} 100 nM), 3 ATP, 10 phosphocreatine, 10 U/ml creatine phosphokinase and 0.2% (w/v) BSA.

Preparation of dispersed submandibular acini. Male (150-200 gm) and pregnant (1 week before parturition) female Sprague-Dawley strain rats were obtained from Sasco Laboratories (Omaha, NB). Rat submandibular acini from adult and newborn (one-day-old) rats were isolated by digestion with collagenase and hyaluronidase as previously described (5, 6).

Determination of cytosolic free Ca^{2+} concentration. $[Ca^{2+}]_i$ was determined using the Ca^{2+} sensitive fluorescent indicator fura-2 using a PTI Deltascan 2.060a fluorometer (S. Brunswick, NJ) as previously described (3,7). 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 (3,7) using 224 nM as K_d of fura-2 for Ca^{2+} (8).

Measurement of $^{45}Ca^{2+}$ uptake and release in permeabilized cells. Submandibular acini were permeabilized and $^{45}Ca^{2+}$ uptake and release were determined as previously described (3,7). An ATP-generation system consisting of 6 mM phosphocreatine and 8 U/ml of creatinine phosphokinase was used to maintain ATP concentration in the reaction system (9). The reaction mixture also contained mitochondrial inhibitors oligomycin (10 μM) and antimycin (10 μM) (3).

Determination of $^{45}Ca^{2+}$ extrusion in intact cells. The plasma membrane Ca^{2+}-pump activity was determined by measuring $^{45}Ca^{2+}$ extrusion. Isolated submandibular acinar cells were loaded with $^{45}Ca^{2+}$ by incubation with 1 μCi/ml of $^{45}Ca^{2+}$ at 23°C for 2 hrs. Then, the cells were rapidly centrifuged at 400 g for 15 sec and $^{45}Ca^{2+}$ loading medium discarded. The cells were resuspended in the same medium (37°C) without $^{45}Ca^{2+}$ in the absence or presence of 1 μM acetylcholine at 37°C for 15 min. Aliquots (0.2 ml) were removed and rapidly filtered through a 3 μm Millipore filter at 0, 0.5, 1, 1.5, 2, 3, 5, 8, 10, and 15 min. The filters were washed with 10 ml of ice-cold medium and the cell $^{45}Ca^{2+}$ content was measured by scintillation counting (3,7).

Data presentation and statistics. All results are presented as means ± S.E. of separate experiments using different cell preparations. Comparisons were made using the unpaired Student t test. P values smaller that 0.05 were considered as significant and stated.

RESULTS

Role of Ca^{2+} release in the enhanced $[Ca^{2+}]_i$ response. The secretagogue-induced initial increase of the transient $[Ca^{2+}]_i$ elevation is mainly produced by Ca^{2+} release from the IP_3-sensitive store in submandibular acinar cells (10,11). As shown in Fig.1, exposure of permeabilized cells to IP_3 (5 μM) elicited a rapid release of preloaded $^{45}Ca^{2+}$, which was similar in both cells of adult and newborn rats. This suggests that the Ca^{2+} release mechanism, i.e., the IP_3-receptors or Ca^{2+} channels, are fully functional in the cells of immature glands and that the enhanced Ca^{2+} response to muscarinic stimulation is not due to increased Ca^{2+} release from the IP_3-sensitive store.

Function of the endoplasmic Ca^{2+} pump. Another possible mechanism responsible for the enhanced increase in $[Ca^{2+}]_i$ in cells of newborn rats is that the function of the Ca^{2+} pump present in the ER and responsible for the reuptake of Ca^{2+} into the store is reduced in the cells of newborn rats. To examine this possibility, an ER Ca^{2+}-ATPase inhibitor thapsigargin

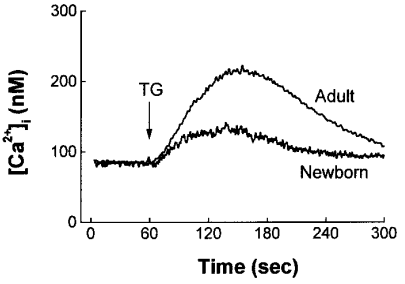

FIG. 2. Increase in cytosolic free Ca^{2+} concentrations in response to thapsigargin. Fura-2-loaded submandibular acinar cells of adult (*Adult*) and newborn (*Newborn*) rats were exposed to 3 μM thapsigargin (*TG*) at the time indicated by the arrow. Each trace is a representative response from separate experiments. Adult, n = 14; newborn, n = 22.

(TG) was used to release Ca^{2+} from the Ca^{2+}-pump-dependent Ca^{2+} store. In the absence of extracellular Ca^{2+}, TG (3 μM) elicited a 126% increase in $[Ca^{2+}]_i$ in cells of adult rats (from 99 $\pm$ 3 nM to 224 $\pm$ 10 nM, n = 14), but a significantly smaller increase (72%) in cells of newborn rats (from 81 $\pm$ 3 nM to 139 $\pm$ 4 nM, n = 22; $P < 0.005$; Fig.2). To corroborate this finding, the release of the radiotracer $^{45}Ca^{2+}$ from permeabilized and tracer-preloaded cells was measured. Inhibition of the ER Ca^{2+} ATPase with TG (3 μM) induced a significantly slower and smaller release of $^{45}Ca^{2+}$ in cells of newborn rats (15.7 $\pm$ 8%, n = 3) than those of adult rats (33.2 $\pm$ 0.6%, n = 5; $P < 0.05$; Fig.3). These results suggest that the function of the endoplasmic Ca^{2+} pump is markedly lower in the cells of the newborn animals.

Function of the PM Ca^{2+} pump. Since increased cytosolic Ca^{2+} is removed to both intracellular Ca^{2+} stores and to the extracellular environment, the function of the PM Ca^{2+} pump was next examined. Extrusion of $^{45}Ca^{2+}$ in cells of mature glands was 22.9 $\pm$ 11% in 15 min (n = 3) in the absence of stimulation (Fig.4A). $^{45}Ca^{2+}$ efflux in the cells of newborn rats was slightly less under the same conditions (15.3 $\pm$ 8% in 15 min (n = 3; Fig.4A). $^{45}Ca^{2+}$ extrusion was potentiated by 1 μM ACh (37.9 $\pm$ 7% in 15 min, n = 3) in cells of adult rats (Fig.4B), but was not enhanced (8.9 $\pm$ 3.1%, n = 4) in cells of newborn rats (Fig.4B). The extrusion of $^{45}Ca^{2+}$ in the presence of ACh was significantly less in cells of newborn rats ($P < 0.01$) than in cells of adult rats, suggesting that the PM Ca^{2+} pump is functionally underdeveloped in newborn animals.

DISCUSSION

Several Ca^{2+} transport mechanisms are involved in the regulation of cytosolic free Ca^{2+} levels. A rapid Ca^{2+} release from the IP_3-sensitive Ca^{2+} store via Ca^{2+} channels in response

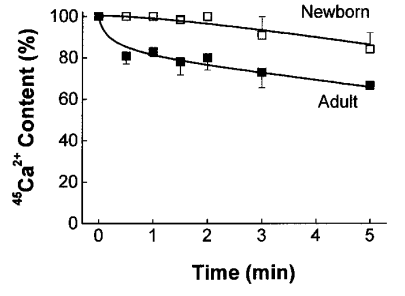

FIG. 3. $^{45}Ca^{2+}$ release induced by thapsigargin. Isolate submandibular acinar cells from adult (*Adult*) and newborn (*Newborn*) rats were permeabilized with saponin and loaded with the radiotracer $^{45}Ca^{2+}$ for 30 min. After loading, the cells were exposed to 3 μM thapsigargin at the time point 0 min. Adult, n = 5; newborn, n = 3.

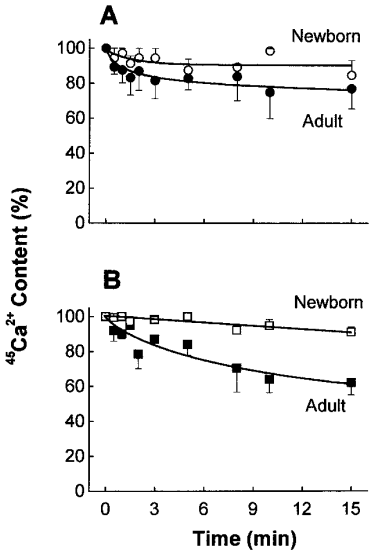

FIG. 4. $^{45}Ca^{2+}$ extrusion in cells preloaded with the radiotracer. Isolated submandibular acinar cells from adult (*Adult*) and newborn (*Newborn*) rats were loaded with the radiotracer $^{45}Ca^{2+}$ for 2 hrs. After loading, the cells were stimulated with 1 μM acetylcholine (B) or vehicle (A) at the time point 0 min. Panel A: $^{45}Ca^{2+}$ extrusion in the absence of stimulation. Adult, n = 3; newborn, n = 3. Panel B: $^{45}Ca^{2+}$ extrusion stimulated with acetylcholine. Adult, n = 5; newborn, n = 4.

to receptor stimulation contributes to the initial increase in $[Ca^{2+}]_i$ (12,13). Immediately after receptor stimulation and Ca^{2+} release, a Ca^{2+} influx from the external environment is activated, yet the activation mechanism of this process is still poorly understood (14). The elevated Ca^{2+} is rapidly removed primarily by two ATP-requiring transport systems, Ca^{2+} re-uptake into the Ca^{2+} store by the ER Ca^{2+} pump and Ca^{2+} extrusion by the PM Ca^{2+} pump. Recent studies (15) indicate these two Ca^{2+} removing processes are strongly activated by receptor occupation.

This study provides evidence that removal of Ca^{2+} from the cytosol is not as effective in submandibular cells of newborn rats primarily as a result of reduced reuptake into the Ca^{2+} store and reduced extrusion through the plasma membrane. In some cell types, such as neurons and muscle, the Na^+/Ca^{2+} exchanger participates in the Ca^{2+} removing process (16,17). However, in salivary cells, the Na^+/Ca^{2+} exchanger seems not to play an important role (18) and Ca^{2+} removal is mainly achieved by the ER and PM Ca^{2+} pumps. The ER Ca^{2+} pump of the cells of newborn rats appears less well developed or responsive. This is demonstrated by two sets of data from experiments with fura-2 and with radiotracer. In fura-2 experiments, inhibition of the ER Ca^{2+} ATPase with thapsigargin (TG) triggered a large release of Ca^{2+} in cells of adult rats. It has been well documented that TG releases Ca^{2+} from the ATP-dependent Ca^{2+} store (19). In contrast, the same treatment caused a significantly smaller release of Ca^{2+} in the cells of newborn rats. Similar results were observed in radiotracer experiments. Exposure of permeabilized and $^{45}Ca^{2+}$-preloaded cells to TG caused only a slow and minimal release of the radiotracer in cells of newborn rats, while the same treatment induced a significant release of $^{45}Ca^{2+}$ in cells of adult rats. These results clearly indicate that the ER Ca^{2+} pump is less affected by TG in cells of newborn rats. This can occur if the pumps are either less numerous or less sensitive to the inhibitor. In addition, this study also provides evidence for a reduced function of the PM Ca^{2+} pump. $^{45}Ca^{2+}$ extrusion was more sluggish and smaller in cells of newborn rats than in cells of adult rats under both unstimulated and ACh-stimulated conditions

(Fig.4). These results indicate that the two major mechanisms for the removal of excess cytosolic Ca^{2+} are not fully developed in cells of early postnatal rats.

Other mechanisms of Ca^{2+} mobilization seem to be well developed in cells of newborn rats. First, the Ca^{2+} release induced by IP_3 is comparable to that seen in mature cells (Fig.1) when studied in permeabilized cells preloaded with the isotopic tracer. Second, Ca^{2+} influx from the extracellular environment was comparable in both cell types (not shown). It is apparent that the enhanced $[Ca^{2+}]_i$ response to acetylcholine is primarily the result of a reduced ability to remove the excess Ca^{2+} by Ca^{2+}-ATPases.

The physiological significance of the larger $[Ca^{2+}]_i$ response to muscarinic stimulation in cells of immature glands is still unclear. It has been demonstrated that the parasympathetic innervation is necessary for morphological and functional development of the major salivary glands (20). Since Ca^{2+} plays a critical role in cell growth and differentiation, it is likely that this enhanced Ca^{2+} response is crucial for development of the submandibular gland.

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