Regulation and identity of intracellular calcium stores involved in membrane cross talk in the early distal tubule of the frog kidney

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Regulation and identity of intracellular calcium stores involved in membrane cross-talk in the early distal tubule of the frog kidney. Am J Physiol Renal Physiol 286: F1219–F1225, 2004. First published March 30, 2004; 10.1152/ajprenal.00255.2003.—The early distal tubule (EDT) of the frog nephron, similar to its mammalian homolog the thick ascending limb of Henle’s loop, actively reabsorbs NaCl. This salt reabsorption, coupled with a low transepithelial water permeability, results in dilution of the tubule fluid, leading to the generic name for these nephron regions, the diluting segment. NaCl reabsorption is energized by the basolateral Na⁺–K⁺-ATPase, which maintains a low intracellular Na⁺ concentration ([Na⁺]) and establishes a favorable chemical gradient for Na⁺ uptake from the luminal fluid. The movement of Na⁺ across the apical membrane is coupled to that of Cl⁻ and K⁺ via an electroneutral cotransporter. Working in concert, these two mechanisms complete the transepithelial transport of Na⁺. The basolateral passage of Cl⁻ is coupled to that of K⁺ via either cotransport or parallel movement through channels. The K⁺ that exits the cell via the basolateral membrane is immediately available for reuptake via the Na⁺–K⁺-ATPase. In this manner, K⁺ recycles across the basolateral membrane and acts as a substrate supporting Na⁺ efflux from the cell. Peculiar to this nephron segment, K⁺ also recycles across the apical membrane. After its absorption from the lumen, K⁺ reenters the tubule fluid via an apical channel, where it is once again taken up by the electroneutral NKCC1 cotransporter (17). This apical recycling is mandatory for the continued reabsorption of NaCl, because the luminal delivery via the glomerular filtrate of K⁺ to the diluting segment is not of itself sufficient to sustain NaCl reabsorption; mutations of the apical K⁺ channels in humans lead to the salt-wasting symptoms of Bartter’s syndrome (29). Thus the uptake of NaCl is determined by the availability of luminal K⁺ (16) and is obligatorily coupled to its movement on the cotransporter. Furthermore, the secretory flux of K⁺ via the apical channels is the rate-limiting step in NaCl absorption and is the principal regulator of salt absorption in this segment (16, 17).

Previous work demonstrated an indirect link between the activity of the apical K⁺ channels and cytosolic Ca²⁺ concentration ([Ca²⁺]). The activity of the apical K⁺ channels is directly regulated by the intracellular pH, where raising pH increases channel activity (23). Intracellular pH is itself predominantly determined by the activity of the Na⁺/H⁺ exchangers located on the basolateral membrane (8). Inhibition of the apical cotransporters by the loop-acting diuretic furosemide results in a rise in cytosolic [Ca²⁺] and is associated with increased activity of the apical K⁺ channels. Furosemide inhibits Cl⁻ uptake on the apical cotransporter and results in a rapid fall in intracellular [Cl⁻]. This fall in intracellular [Cl⁻] results in Ca²⁺ release from intracellular stores, thereby elevating cytosolic [Ca²⁺] (7). The increase in intracellular [Ca²⁺] activates the basolateral Na⁺/H⁺ exchangers, via calmodulin, and the consequent intracellular alkalization directly upregulates apical K⁺ channel activity, increasing the availability of luminal K⁺ (9). This complex series of regulatory steps can be seen as a mechanism to maintain Na⁺ absorption in the face of reduced Na⁺ delivery to the diluting segment. Therefore, a rise in cytosolic Ca²⁺ is central to membrane cross talk, pump-leak coupling, and, therefore, the regulation of salt absorption in this nephron region.

The principal question remaining from the experiments described above is the source of intracellular Ca²⁺ involved in this feedback response. Previous work suggested that this was the endoplasmic reticulum (ER), because depletion of the


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intracellular Ca\(^{2+}\) stores with the SERCA-specific inhibitor 2,5-di(tert-butyl) hydroquinone (TBQ) ablated the furosemide-induced rise in intracellular Ca\(^{2+}\) (9). The purpose of the experiments described in this paper was to seek functional evidence for the identity of intracellular Ca\(^{2+}\) storage pools in the renal diluting segment, which may be involved in pump-leak coupling. In this manuscript, we describe experiments designed to elucidate functional calcium storage pools within the amphibian diluting segment. To our knowledge, this is the first time that such experiments have been conducted in native renal tissue. The results show that the predominant pool is the ER and further show modulation of ER calcium content with physiological regulators. These studies provide strong corroborative evidence in support of our earlier findings concerning the ER as the source of calcium in pump-leak coupling in the amphibian EDT.

METHODS

Preparation of EDT Segments for Monitoring Compartmentalized Probe

Frogs (\emph{Rana temporaria}) of either sex were kept in tap water at 4°C. Animals were stunned by concussion, and the brain and spinal cord were destroyed by pithing in accordance with the UK legislature. The kidneys were removed, cut into 1-mm sections, and stored in ice-cold Leiboitz solution containing 140 mM NaCl, 2 mM KCl, 10 mM Ca\(^{2+}\), 10 mM Mg\(^{2+}\), 15 mM HEPES, 10 mM EGTA, and 10 mM MgCl\(_2\) and 10 mM HEPES, titrated to pH 7.4 with NaOH. The "intracellular" solution had the following composition (in mM): 88 K- Gluconate, 12 NaCl, 2 MgSO\(_4\), 1 free [Mg\(^{2+}\)], 4 Ca(NO\(_3\))\(_2\), 200 mM free [Ca\(^{2+}\)], 10 mM EGTA, and 10 mM HEPES, titrated to pH 7 with KOH/glucuronic acid lactone as appropriate.

Where required, additions to the intracellular solution were made from the following stock solutions (unless otherwise stated, all chemicals were obtained from Sigma, Poole, UK, and dissolved in water): 1 mM or 1 M K-ATP, 0.5 M K-ADP (FLUKA), 1 M KH\(_2\)PO\(_4\), 0.5 M succinic acid, 1 M Ca(NO\(_3\))\(_2\), 50 mg/ml saponin, 100 mM MnCl\(_2\), 10 mM rotenone in DMSO, 10 mM FCCP in DMSO, 10 mM TBQ in DMSO, 10 mM IP\(_6\), 100 mg/ml heparin, 10 mM ionomycin in DMSO, and 5 mg/ml oligomycin in DMSO (mix of A, B, and C).

Fluorescence Microscopy

The compartmentalization within intracellular organelles of the low-affinity calcium-sensitive probe mag-fura 2-AM (2 Molecular Probes, Leiden, The Netherlands) has been exploited here to monitor changes in intrastore Ca\(^{2+}\) following permeabilization of the basolateral membrane. Although the fluorescent probe chosen is also sensitive to Mg\(^{2+}\), it is of much lower affinity than that for Ca\(^{2+}\) and has been shown to be insensitive to Mg\(^{2+}\) in physiological type intracellular buffers (20, 33). The basic recording system (Newcastle Photometrics) has been described in detail previously (9). Briefly, mag-fura-2 was alternately excited at 350 and 380 nm for 500 ms, and emitted fluorescence is clearly confined to the cytosol, with some punctuate juxta-nuclear located regions that may represent compartments with high esterase activity or very low Ca\(^{2+}\) content (see DISCUSSION). Scale bar: 30 μm.

Data Analysis and Statistics

Data are presented as continuous experimental recordings with time on the x-axis and 350/380 ratio on the y-axis, or as mean steady-state ratio values ± SE. No attempt has been made to calibrate the intrastore signals because a mixed population of Ca\(^{2+}\) stores invalidates such an approach (21). On the other hand, it was necessary to ascertain that the probe was sensitive to changes in Ca\(^{2+}\) following permeabilization, which was achieved by exposure to a Ca\(^{2+}\) iono-
phore and different levels of Ca\textsuperscript{2+}: after incubation with 5 μM ionomycin, the 350/380 fluorescence ratio was 0.26 ± 0.003 in Ca\textsuperscript{2+}-free and 1.07 ± 0.01 (n = 3) in a 5 mM Ca\textsuperscript{2+} solution, respectively. All starting ratios were above this minimum and all peak fluorescence ratios fell below the maximum. Additionally, changes in fluorescence while adding intracellular regulators occurred rapidly, suggesting that the probe was effectively reporting changes in Ca\textsuperscript{2+}, even with very low levels of substrates (e.g., see ATP and TBQ dose responses in RESULTS), without any appreciable delay other than that caused by the lag in solution exchange.

Statistical analysis was carried out in Excel (Microsoft) using paired or unpaired t-tests as appropriate. Where appropriate, ANOVA analysis was performed with Minitab (Minitab, State College). Significance was assumed at the 5% level. Sigma Stat (Jandel Scientific) was used for nonlinear curve fitting in the determination of \( K_d \) values.

RESULTS

Factors Modulating Ca\textsuperscript{2+} Uptake into the Internal Store

ATP and Ca\textsuperscript{2+}. Addition of ATP to the bath solution in the presence of 200 nM Ca\textsuperscript{2+} promoted a dose-dependent increase in the 350/380 ratio, consistent with the movement of Ca\textsuperscript{2+} into the internal store (Fig. 2). The mean data from these experiments are summarized in the inset and are described well by saturation kinetics with a \( K_d \) of 2.6 ± 0.66 μM (n = 5, \( P < 0.05 \)). The degree of store filling also depended on the Ca\textsuperscript{2+} concentration of the bath fluid: in the presence of a saturating concentration of ATP (0.1 mM), incremental increases in the 350/380 ratio were seen on the addition of Ca\textsuperscript{2+} to the bathing solution over the range of 10 nM to 10 μM (data not shown) and reflect the ability of the store to accumulate Ca\textsuperscript{2+} over a wide concentration range. These results suggest that Ca\textsuperscript{2+} is moved against its concentration gradient at the expense of ATP and that the transport mechanisms operate over a wide range of intracellular Ca\textsuperscript{2+} concentrations.

SERCA inhibition. Ca\textsuperscript{2+}-transporting ATPases of the SERCA family are sensitive to TBQ, which is a specific inhibitor of ER Ca\textsuperscript{2+} pumps (10). Exposure of permeabilized tubules to ATP in the presence of 10 μM TBQ gave no change in the 350/380 ratio, consistent with the abolition of SERCA pump activity (350/380 ratio: control 0.28 ± 0.005; TBQ 0.28 ± 0.005, n = 6, \( P > 0.05 \)). Increases in the 350/380 ratio occurred following stepwise reduction in the bath TBQ concentration from 10 to 3, 1, 0.3, and 0 μM TBQ (Fig. 3). The mean data from six tubules (inset) illustrate the progressive relief from pump inhibition as the TBQ concentration is decreased (n = 6, \( P < 0.05 \)). These results confirm the presence of a SERCA pump as an uptake pathway.

Fig. 2. Store loading in response to low levels of ATP. Filling of the internal stores was dose dependent (as shown by the recording under the horizontal bars) and appeared to have saturated in response to 0.1 mM ATP. Inset: mean data from 5 tubules. Solid line is best fit to an equation representing saturation kinetics, yielding a \( K_d \) of 2.6 ± 0.66 μM. R.U., ratio units.

Fig. 3. Attenuation of store loading by the SERCA-specific inhibitor 2,5-di(\textit{tert}-butyl) hydroquinone (TBQ). TBQ was added to the bath (before the addition of ATP), and its concentration was decreased sequentially as indicated by the numbers over the horizontal bars. Inset: mean data from 6 tubules. Solid line connects points and has no theoretical significance.
Factors Mediating Loss of Ca\(^{2+}\) from the Internal Store

**Passive Ca\(^{2+}\) leak.** In the steady state, the store Ca\(^{2+}\) content reflects equal rates of influx and efflux, a change in either of these processes will alter the store Ca\(^{2+}\) equilibrium. In an attempt to determine the pathway of the passive leak, the effect of heparin, an IP\(_3\) receptor antagonist (14), on unstimulated store Ca\(^{2+}\) release was examined. Permeabilized tubules were exposed to 0.1 mM ATP to fill the internal store, and ATP was subsequently removed from the bath solution to reveal the passive loss of store Ca\(^{2+}\) (Fig. 4A). Calcium leak from the store followed an exponential time course that was unaffected by the addition of the IP\(_3\) receptor blocker heparin (100 \(\mu\)g/ml, a concentration that has been previously determined to cause complete reversal of IP\(_3\)-mediated release, \(n = 6\); Fig. 4B); time constant for 350/380 ratio decline following removal of ATP: control 1,750 ± 690 s, heparin 1,633 ± 529 s, \(n = 5\), \(P > 0.05\) (Fig. 4A). Loss of Ca\(^{2+}\) from the store was also apparent when Ca\(^{2+}\) was removed from the bath (data not shown). It appears therefore that the presence of both ATP and Ca\(^{2+}\) is required to maintain store Ca\(^{2+}\) load.

**Ryanodine receptor-mediated efflux: effect of cADPR.** The effect of the pyridine nucleotide metabolite cADPR in releasing Ca\(^{2+}\) through the ryanodine receptor has been described in sea urchin egg homogenates (11). cADPR was without effect following store filling after application to the bathing medium (350/380 ratio; ATP 0.35 ± 0.02, cADPR 0.35 ± 0.01, \(n = 6\), \(P > 0.05\)). This observation is consistent with previous observations that the application of ryanodine to the bath was also without effect on the 350/380 ratio (7). IP\(_3\), however, was still able to induce Ca\(^{2+}\) release (350/380 ratio: ATP 0.35 ± 0.01, IP\(_3\) 0.28 ± 0.004, \(n = 6\)).

**IP\(_3\)-sensitive Ca\(^{2+}\) efflux.** Store filling was promoted by the addition of 2 mM ATP to the bath, a concentration known to maximize the open probability of the IP\(_3\) receptor (3), before the addition of IP\(_3\) to the bath. The efflux of Ca\(^{2+}\) was clearly dependent on the concentration of IP\(_3\) (Fig. 5), with an apparent \(K_d\) of 1 \(\mu\)M. (see inset) and showing saturation at 3 \(\mu\)M.

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**Fig. 4.** Passive leak of store Ca\(^{2+}\). A: passive leak of store Ca\(^{2+}\) as revealed by heparin, an IP\(_3\) receptor antagonist. A typical experiment in which ATP (0.1 mM) was present throughout the period is indicated by the bar. On removal of ATP (but with a bath [Ca] of 200 nM), the passive leak is exposed. Heparin (100 \(\mu\)g/ml) was added during the period of passive store leak for the duration of the horizontal bar. Trace representative of 5 separate experiments. B: heparin (100 \(\mu\)g/ml) is an effective antagonist of the effects of IP\(_3\).

**Fig. 5.** Release of store Ca\(^{2+}\) by IP\(_3\). Store filling was induced with 2 mM ATP, which was present throughout the remainder of the experiment. IP\(_3\) was added for the periods indicated by the horizontal bars and at the indicated concentrations. *Inset:* mean data from 6 tubules. Solid line is best fit to Michaelis-Menten equation with an apparent \(K_d\) of 1 \(\mu\)M.
Because virtually all Ca\(^{2+}\) was released with 3 \(\mu\)M IP\(_3\), this concentration was used in all subsequent experiments. Furthermore, the IP\(_3\) receptor antagonist heparin (100 \(\mu\)g/ml) abolished the effect of IP\(_3\) on store calcium (see Fig. 4B as previously discussed, \(n = 6\)). IP\(_3\) therefore appears to be an important regulator of store Ca\(^{2+}\) content.

**Evidence for an IP\(_3\)-Independent Store**

Permeabilized tubules were exposed to ionomycin in the absence of ATP, i.e., without store loading. There was no effect on the 350/380 ratio following this intervention (350/380 ratio: control 0.27 ± 0.003; ionomycin 0.27 ± 0.003, \(n = 7\), \(P > 0.05\)). As previously shown, after Ca\(^{2+}\) loading with ATP, IP\(_3\) (3 \(\mu\)M) promoted a rapid fall in store Ca\(^{2+}\) that represented 58 ± 5% of the total release. However, addition of ionomycin promoted a further reduction in the 350/380 ratio, consistent with a release from an IP\(_3\)-insensitive store that was 42 ± 5% of the total release (\(n = 6\); Fig. 6A). It is possible that these data represent Ca\(^{2+}\) release from a single compartment that occurs with two different time courses. However, we excluded this time-dependent effect on Ca\(^{2+}\) within the store because Fig. 6, A and B, shows that store Ca\(^{2+}\) decreases only after the addition of ionomycin, despite differences in the time course of initial application of the compound. Furthermore, Fig. 5 demonstrates that steady-state levels of store Ca\(^{2+}\) are only altered by additions of IP\(_3\) and not by any effect of time (total exposure time to IP\(_3\) > 5 min).

We examined the possibility that the IP\(_3\)-insensitive pool, unmasked by ionomycin, represented storage by mitochondria. The ATP synthase inhibitor oligomycin (10 \(\mu\)g/ml) was added to the bath, following release by IP\(_3\), but was found to be without effect on store Ca\(^{2+}\) content, whereas the addition of ionomycin (5 \(\mu\)M) promoted a further reduction in the 350/380 ratio (\(n = 7\); Fig. 6B). A second series of experiments was performed in which the mitochondrial membrane potential (\(\Delta\psi_m\)), and hence the driving force for Ca\(^{2+}\) entry, was maintained by operation of the respiratory chain. This was achieved by adding ATP (2 mM) to the bath alongside “respiratory substrates” that included ADP (1 mM), KH\(_2\)PO\(_4\) (1 mM phosphate), and a Krebs or TCA cycle intermediate, succinate (substrates of glycolysis would have been ineffective in this preparation because glycolysis occurs in the cytosol, and we were unable to rely on the integrity of the constituent enzymes in the permeabilized preparation, which may have been washed away or otherwise compromised). We relied on atmospheric gases, dissolved in the bath solution, to supply the preparation with oxygen. Figure 7A shows that such a cocktail of substrates promoted an increase in the 340/380 ratio, consistent with Ca\(^{2+}\) uptake, from 0.34 ± 0.02 to 0.38 ± 0.01 (\(n = 7\)). This increase in Ca\(^{2+}\) was unaffected by the addition of the electron acceptor compounds described above, whereas IP\(_3\) was able to induce Ca\(^{2+}\) release.

**Fig. 7. Identification of the IP\(_3\)-insensitive store**

A: in vivo mitochondria are likely to be exposed to respiratory substrates in addition to ATP. Exposure to this combination of compounds induced Ca\(^{2+}\) uptake that was insensitive to inhibition of the respiratory chain by rotenone but could be released by IP\(_3\). B: exposure to respiratory substrates after ATP-induced uptake does not promote further Ca\(^{2+}\) uptake; FCCP was without effect on store Ca\(^{2+}\), whereas IP\(_3\) was able to induce Ca\(^{2+}\) release.
transport chain complex 1 inhibitor rotenone (1 \( \mu \)M) (350/380 ratio: substrates 0.38 ± 0.009; rotenone 0.38 ± 0.001, \( n = 7 \), \( P > 0.05 \)) but was reversed by the addition of IP3 (3 \( \mu \)M), suggesting that Ca\(^{2+} \) was entering the ER/IP3-sensitive store. Similarly, no further increase in store Ca\(^{2+} \) was observed when permeabilized tubules were exposed to respiratory substrates after Ca\(^{2+} \) accumulation had been induced with ATP (2 \( \mu \)M) (350/380 ratio: ATP 0.37 ± 0.01; ATP and substrates 0.38 ± 0.01, \( n = 6 \), \( P > 0.05 \); Fig. 7B), presumably, again, into the ER/IP3-sensitive store. Consistent with this, FCCP (5 \( \mu \)M) was without effect on accumulated Ca\(^{2+} \) (350/380 ratio: ATP 0.37 ± 0.01; FCCP 0.38 ± 0.01; analysis by ANOVA with post hoc Dunnet’s comparison), whereas IP3 was able to promote Ca\(^{2+} \) mobilization.

**DISCUSSION**

The subcellular distribution and regulation of intracellular Ca\(^{2+} \) stores have been identified in a variety of cell types that include isolated gastric glands (19), BHK-21 fibroblasts (20), pancreatic acinar cells (33), and lens cells (5). Typically, store filling is via an ATP-dependent Ca\(^{2+} \) pump of the SERCA family, whereas emptying of the store is regulated through an IP3 receptor regulator cADPR. This is in agreement with our earlier work, in which addition of ryanodine directly to the bathing medium was without effect on store Ca\(^{2+} \) (7). Therefore, it is unlikely that there is a mixed expression of intracellular Ca\(^{2+} \) release channels in the EDT region. In vivo, it is therefore conceivable that release from the ER may well be mediated by IP3. In human fibroblasts, it has been shown that a reduction in extracellular Na\(^{+} \) promotes the formation of inositol polyphosphates and a release of Ca\(^{2+} \) (30). Similarly furosemide, which causes an abrupt fall in intracellular Na\(^{+} \) in the EDT (27), also results in the release of store Ca\(^{2+} \), raising the possibility that this mechanism is mediated by IP3.

Additional Ca\(^{2+} \) release following store loading with ATP was induced by the addition of ionomycin to the bath following store depletion with IP3. There are several possibilities to explain this observation. 1) This represents anatomic homogeneity but functional heterogeneity (i.e., not all the Ca\(^{2+} \) within the store is released) within the IP3-sensitive pool. This may explain the ATP-dependent uptake but the lack of effect of ionomycin in the absence of store loading. An example of such a store would be the nuclear envelope, which is not only continuous with the ER (13) but has been shown to have functional Ca\(^{2+} \) uptake (13) and release pathways (12, 22, 31).

2) It is also conceivable that this result represents a differential expression of IP3 receptors to the ER membrane and as such Ca\(^{2+} \) release in the presence of IP3 occurs from the part of the store with the highest level of receptor expression. We have been unable to image the patterns of release and are therefore unable to resolve this possibility, but it is tempting to speculate that focal Ca\(^{2+} \) release could target the Ca\(^{2+} \) signal to discrete calmodulin-rich areas to accomplish activation of the sodium hydrogen exchanger. 3) This observation may represent the activity of a spatially inhomogeneous store such as mitochondria (19) or another endomembranous compartment, e.g., lysosomes (18). Under the current conditions, the fluorescence signal is unaffected by addition of the protonophore FCCP, the actions of which are not confined to mitochondria, but to all compartments across which a proton gradient is maintained (see Fig. 6B and also Ref. 25). This has two implications. It suggests 1) that lysosomes do not make a significant contribution to Ca\(^{2+} \) cycling in the frog EDT and rules out any artefactual, pH-induced, fluorescence changes that result from the activity of proton-transporting ATPases on the lysosomal membrane and 2) that mitochondria do not participate directly in the uptake and release of Ca\(^{2+} \) under these conditions. This may not be too surprising given that the affinity of the Ca\(^{2+} \) uniporter is in excess of 1 \( \mu \)M (2), which is an order of magnitude higher than the Ca\(^{2+} \) levels used in the present study.

The data presented in this investigation rely on the compartmentalization into cellular pools of a low-affinity Ca\(^{2+} \)-sensitive probe. In our system, mag-fura 2 appears to respond to large changes in Ca\(^{2+} \) (from 0 to 5 mM), equilibrated across
the organelle membranes using ionomycin, which produces concomitantly large changes in the fluorescence ratio that would be well in excess of the levels of Ca$^{2+}$ anticipated to reside within the stores (4). However, signal responses from this preparation may be limited because of 1) a low pumping activity with low levels of “resting” Ca$^{2+}$, 2) washout of cellular regulators during permeabilization that modulate the activity of store-filling mechanisms, or 3) that “silent” compartments bias the fluorescence signal in favor of probe trapped in pools where Ca$^{2+}$ resides below the detection limit of the probe (~5 µM) (21). This occurs because fluorescence ratio increases rely on quenching of fluorescent probe by Ca$^{2+}$. Therefore, a probe trapped equally in two compartments will return an average fluorescence signal of the two pools; the compartment containing high Ca$^{2+}$ will have a quenched signal compared with the compartment containing low Ca$^{2+}$. Thus a proportionately larger part of the signal is composed of fluorescence from the low-Ca$^{2+}$ compartment. If Ca$^{2+}$ is not moved into this pool at all during uptake, then changes in the fluorescence signal will arise from movements into and out of just one store, which will suppress the magnitude of the observed changes (21). Due to these complications, the current data remain uncalibrated.

In summary, frog EDT cells maintain a constant internal store Ca$^{2+}$ content at the expense of ATP. The availability of ATP is unlikely to be rate limiting to Ca$^{2+}$ accumulation, given the high affinity of the Ca$^{2+}$ uptake mechanism relative to the expected intracellular concentration of ATP. Ca$^{2+}$ is released via the IP$_3$ receptor, and not the ryanodine receptor, whereas the sensitivity toward the SERCA-specific inhibitor TBQ and the endogenous releasing agent IP$_3$ strongly implicates the ER as the major site of Ca$^{2+}$ storage within the EDT.

GRANTS

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REFERENCES


