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

Mark R. Fowler, Gordon J. Cooper, and Malcolm Hunter

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, is a site of major ion uptake and sets the stage for the production of concentrated urine by the nephron. The EDT, analogous to the thick ascending limb of the mammalian kidney, is the site of limiting sodium absorption that establishes the gradient for water excretion. The EDT also reabsorbs sodium and chloride, which is regulated by the intracellular pH, where raising pH increases channel activity (23). Intracellular pH is itself primarily 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, therefore 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 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 primarily 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, therefore 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

Address for reprint requests and other correspondence: M. Hunter, School of Biomedical Sciences, Worsley Bldg., Univ. of Leeds, Leeds, West Yorkshire LS2 9NQ, UK (E-mail: m.hunter@leeds.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajprenal.org 0363-6127/04 $5.00 Copyright © 2004 the American Physiological Society
intracellular Ca\(^{2+}\) stores with the SERCA-specific inhibitor 2,5-ditert-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 calcium 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 (*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 had the following composition (in mM): 88 K-gluconate, 12 NaCl, 2 MgSO\(_4\), 1 free [Mg\(^{2+}\)], 10 EGTA, and 10 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

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\(^2+\): after incubation with 5 μM ionomycin, the 350/380 fluorescence ratio was 0.26 ± 0.003 in Ca\(^2+\)-free and 1.07 ± 0.01 \((n = 3)\) in a 5 mM Ca\(^2+\) 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\(^2+\), 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\(^2+\) Uptake into the Internal Store

**ATP and Ca\(^2+\).** Addition of ATP to the bath solution in the presence of 200 nM Ca\(^2+\) promoted a dose-dependent increase in the 350/380 ratio, consistent with the movement of Ca\(^2+\) 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\(^2+\) 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\(^2+\) 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\(^2+\) over a wide concentration range. These results suggest that Ca\(^2+\) is moved against its concentration gradient at the expense of ATP and that the transport mechanisms operate over a wide range of intracellular Ca\(^2+\) concentrations.

**SERCA inhibition.** Ca\(^2+\)-transporting ATPases of the SERCA family are sensitive to TBQ, which is a specific inhibitor of ER Ca\(^2+\) 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 \((350/380 \text{ ratio: control } 0.28 \pm 0.005; \text{ TBQ } 0.28 \pm 0.005, n = 6, P > 0.05)\). 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 \((\text{inset})\) 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.
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 \pm 690 s, heparin 1,633 \pm 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 \pm 0.02, cADPR 0.35 \pm 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 \pm 0.01, IP\(_3\) 0.28 \pm 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.

![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\(_3\).](http://ajprenal.physiology.org/)

![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.](http://ajprenal.physiology.org/)
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 oligomycin (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

---

**Fig. 6.** Ca\(^{2+}\) store mobilization by ionomycin and IP\(_3\). A and B: stores were loaded with 2 mM ATP, which was present throughout the experiment. A: IP\(_3\) (3 \(\mu\)M) was added during the period indicated by the horizontal bar and promoted a release of store Ca\(^{2+}\). Tubules were also exposed to ionomycin (5 \(\mu\)M) for the time indicated, which also promoted a release of store Ca\(^{2+}\). B: after addition of IP\(_3\), the tubule was exposed to oligomycin (10 \(\mu\)g/ml) for the period indicated by the horizontal bar. Oligomycin failed to elicit Ca\(^{2+}\) release, although there was a further release of Ca\(^{2+}\) in response to ionomycin.

**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 I inhibitor rotenone (1 μM) (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 (5 μM), suggesting that Ca2+ was entering the ER/IP3-sensitive store. Similarly, no further increase in store Ca2+ was observed when permeabilized tubules were exposed to respiratory substrates after Ca2+ accumulation had been induced with ATP (2 mM) (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 μM) was without effect on accumulated Ca2+ (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 Ca2+ mobilization.

DISCUSSION

The subcellular distribution and regulation of intracellular Ca2+ 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 Ca2+ pump of the SERCA family, whereas emptying of the store is regulated through an IP3 receptor (19). The presence of these filling and release mechanisms most likely represents Ca2+ storage by the ER (6, 28, 32). Pharmacological manipulation has revealed other Ca2+ pools, such as the mitochondria (19) and lysosomes (18). The following study examined the cellular regulation and functional distribution of intracellular pools capable of storing Ca2+ in the frog EDT, because Ca2+ has been shown to be a key, albeit indirect, regulator of apical K+ channels and hence salt transport.

The ER appears to be a major Ca2+ store within the cells of the diluting segment because Ca2+ accumulation is sensitive to the SERCA-specific blocker TBQ; inhibition of SERCA activity with this compound prevents the global, furosemide-induced increase in intracellular Ca2+ (9). Furthermore, the application of TBQ alone, before store loading, never resulted in a decline in the 350/380 ratio (an effect indicative of Ca2+ loss via the leak pathway), which is consistent with the idea that there is little SERCA activity and thus ER loading following time spent in an ATP-free environment during dye loading. The data display an apparent $K_i$ of 2 μM toward TBQ. This is in good agreement with inhibitory assays on rat liver microsomes (26) that demonstrated a $K_i$ of 1 μM and a maximal inhibitory effect at 10 μM. The depletion of accumulated Ca2+ in the absence of ATP (or Ca2+) is also consistent with the notion that the continued presence of both these substrates is required to maintain a constant luminal Ca2+ concentration and thus the integrity of the pump-leak coupling mechanism. The affinity of Ca2+ uptake toward ATP strongly suggests that ATP is unlikely to be rate limiting in maintaining store Ca2+ content, assuming an intracellular ATP concentration in excess of 0.1 μM (1). Furthermore, we calculated $K_d$ for ATP with respect to the uptake process of 2.6 μM is several orders of magnitude lower than other estimates using a similar technique in gastric epithelial cells (~1.5 mM, see Ref. 19). However, both these estimates are considerably higher than values obtained using COS cell microsomes whereby the $K_{i/2}$ for the formation of a phosphorylated intermediate for SERCA 2b and SERCA3 (the most likely nonmuscle isomers) are 20 and 50 nM, respectively (24). These differences are probably due to the fact that the fluorescence assay represents a global average of all ATP-dependent uptake processes. Similarly, data where Ca2+ transport varied with bath [Ca2+] could be fitted using a $K_d$ of 230 nm. This value is similar to Ca2+ transport values published for SERCA2b, which elicited a $K_d$ of 270 nM (24).

Release of Ca2+ from the ER appears to be dominated by the IP3 receptor; there was no effect of the endogenous ryanodine 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 Ca2+ (7). Therefore, it is unlikely that there is a mixed expression of intracellular Ca2+ 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 Ca2+ (30). Similarly, furosemide, which causes an abrupt fall in intracellular Na+ in the EDT (27), also results in the release of store Ca2+, raising the possibility that this mechanism is mediated by IP3.

Additional Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ release could target the Ca2+ signal to discrete calmodulin-rich areas to accomplish activation of the sodium hydrogen exchanger. 3) This observation may represent the activity of a spatially inhomogenous 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 protonphore 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 Ca2+ 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 Ca2+ under these conditions. This may not be too surprising given that the affinity of the Ca2+ uniporter is in excess of 1 μM (2), which is an order of magnitude higher than the Ca2+ levels used in the present study.

The data presented in this investigation rely on the compartmentalization into cellular pools of a low-affinity Ca2+-sensitive probe. In our system, mag-fura 2 appears to respond to large changes in Ca2+ (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 \(\mu\)M) (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, 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.

REFERENCES

GRANTS

The support of the National Kidney Research Fund is gratefully acknowledged.

REFERENCES