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Fluorescent Measurements of Intracellular Free Calcium in Isolated Toad Urinary Bladder Epithelial Cells

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Summary. Sodium-calcium exchange has been suggested to play a pivotal role in the regulation of cytosolic free calcium (Ca_f) by epithelial cells. Using isolated epithelial cells from the toad urinary bladder, Ca_f has been measured using the intracellular Ca-sensitive fluorescent dyes Fura 2 and Quin 2. Dye loading did not alter cell viability as assessed by measurements of ATP and ADP content or cell oxygen consumption. When basal Ca_f was examined over a wide range of cell dye content (from 0.04 to 180 nmol dye/mg protein) an inverse relationship was observed. At low dye content, Ca_f was 300–380 nM and, as dye content was increased, Ca_f progressively fell to 60 nM. Using low dye content cells, in which minimal alteration in Ca steady state would be expected, the role for plasma membrane Na-Ca exchange was examined using either medium sodium substitution or ouabain. While medium sodium substitution increased Ca_f, prolonged treatment with ouabain had no effect on Ca_f despite a clear increase in cell sodium content. The lack of effect of ouabain suggests that Na-Ca exchange-mediated Ca efflux plays a minimal role in the regulation of basal Ca_f. However, exchange-mediated Ca efflux may play a role in Ca_f regulation when cytosolic calcium is elevated.

Key Words Fura 2 · Quin 2 · *Bufo marinus* · epithelial cells · ouabain · Na-Ca exchange

Introduction

Cytosolic free calcium (Ca_f) has been implicated as a regulator of epithelial sodium transport, presumably by altering luminal Na permeability [37]. The proposed role for Ca regulation predicts that an increase in Ca_f decreases sodium permeability and is based on the observation that sodium transport is reduced by: (i) Ca ionophores in toad [44] and turtle [2] bladders; (ii) maneuvers believed to inhibit cellular Ca efflux mediated by Na-Ca exchange such as reduced serosal Na [15, 24] and addition of either quinidine [3, 24] or ouabain [24, 25]; and (iii) the report that Ca reduces Na permeability in toad bladder plasma membrane vesicles [11]. However, conflicting evidence exists, questioning this simple scheme. Both ADH and theophylline, which stimulate sodium transport, increase ^{45}Ca efflux from intact toad bladder [12] and isolated bladder cells [8], presumably by release of intracellular Ca stores into the cytosol with subsequent efflux across the plasma membrane. Calcium ionophores have also been shown to increase sodium transport in frog skin to a similar extent as the stimulatory action of ADH [5] and in toad bladders, the inhibitory action of Ca ionophores can be reversed by ADH [37]. While ample suggestive evidence for an involvement of Ca_f in transport regulation is available, the regulation of Ca_f in intact cells is ambiguous and specific information regarding changes in Ca_f is minimal.

The plasma membrane Na-Ca exchange has been proposed to play a pivotal role in the regulation of Ca_f [15, 24, 37]. In this scheme, an increase in intracellular sodium reduces the plasma membrane Na gradient available to drive efflux of Ca via Na-Ca exchange resulting in an increased Ca_f. Implicit to this model are the assumptions that (i) the Na-Ca exchange is sensitive to slight changes in cell Na concentration, particularly under conditions in which Ca_f has been suggested to regulate Na transport and cell volume; and (ii) that the change in Ca efflux is sufficient to change Ca_f. The latter point assumes that other Ca transport mechanisms, including plasma membrane Ca-ATPase, endoplasmic reticulum Ca uptake and mitochondrial Ca uptake, play a lesser role in regulation of Ca_f.

The recent availability of intracellular Ca-sensitive fluorescent dyes [16, 40] provides a method for directly evaluating the regulation of Ca_f. In the present study, the role of Na-Ca exchange in the maintenance of Ca_f was examined using both Fura 2 and Quin 2 in isolated toad bladder cells. In the course of these studies a significant dye-Ca_f interaction was observed. The nature of the interaction has been partially characterized and appears to be related to dye-induced alterations in cell Ca handling.

Materials and Methods

Isolated Bladder Cell Preparation

Bladders from 3–6 doubly pithed toads (*Bufo marinus*) were perfused *in situ* via the abdominal aorta with an amphibian Ringer solution containing (in mM) 102 NaCl, 3.5 KCl, 3 NaH_2PO_4, 1 $MgSO_4$, 0.9 $CaCl_2$, 5 glucose, pH 7.4 until free of blood. The bladders were removed and incubated for 1 hr at 25°C in Ringer solution to which 0.6% (wt/vol) dialyzed dextran and 1 mg/ml collagenase (type 1a, low trypsin, Sigma Chemical Co.) were added. Following collagenase incubation, the mucosal surface was scraped with a microscope slide to liberate the epithelial cells. Collected cells were diluted in Ringer + dextran (R + D) and centrifuged at 750 × *g* for 5 min at 4°C. Pellets were suspended in R + D, layered onto 60% (vol/vol) Percoll containing 96 mM NaCl, 3 mM NaH_2PO_4, pH 7.4–7.6, and centrifuged at 750 × *g* for 5 min to remove debris and trace red blood cells. Percoll centrifugation was repeated two times after which the cells were collected and washed in R + D. The resulting pellets were resuspended in R + D and divided into polypropylene flasks to which either Fura 2 AM (0.5–10 μM) or Quin 2 AM (2–100 μM) were added. Controls received equivalent volumes of DMSO alone. Cells were incubated for 15 min to 2 hr at 25°C after which the suspensions were diluted in ice-cold R + D and washed two times. Dye-loaded cells were preincubated at 25°C for 30 min prior to fluorescence measurement.

Fluorescence Measurement

Fura 2 and Quin 2 fluorescence was measured using a custom-built fluorometer, as described below. Excitation light from a 150-W Xenon arc lamp was filtered through a grating monochromator followed by a UG-11 filter and directed to a quartz cuvette placed in a thermostatically controlled (25°C) holder. Cell suspensions, 3 ml final volume, were continuously stirred with a magnetic stir bar. Emitted fluorescence was collected by a fiber optic bundle, passed through a second grating monochromator and directed onto a photomultiplier tube. Fura 2 was excited at 350 nm while Quin 2 was excited at 340 nm. For both dyes the emitted fluorescence was monitored at 510 nm.

Ca_f was determined by a modification of previously reported methods [9, 27, 36, 41, 47]. After recording cell fluorescence for 5 min, (F_1), 5 mM EGTA and 3.3 mM Tris base were added to estimate the amount of extracellular dye in the sample (F_2). In both Fura 2 and Quin 2 studies, the use of EGTA to estimate extracellular dye was confirmed by random comparison of dye dependent fluorescence in parallel suspension supernatants. For Quin 2 studies, additional verification was obtained by examining the effect of $MnCl_2$ (1.5 μM), which quenches 50% of the dye signal [27]. In all comparisons a greater than 95% agreement was observed. Digitonin, 67 μg/ml, was then added to permeabilize the cell membrane and, in the presence of EGTA, reduce dye fluorescence to a minimum value (F_3). After a stable F_3 had been recorded (2–5 min), $CaCl_2$, 5.5 mM, was added to saturate the dye and establish maximal fluorescence (F_4). Parallel recordings of cell autofluorescence were obtained using non-loaded cells and used to correct dye fluorescence recordings.

The value for Ca_f was calculated based on the recorded fractional occupancy of the dye and K_d using the equation

$$Ca_f = K_d \cdot ((F_2 - F_3)/(F_4 - F_1))$$

The K_d for Fura 2 and Quin 2 were determined at 25°C in a buffer containing (in mM) 105 KCl, 20 NaCl, 1 $MgCl_2$, 10 MOPS, pH 7.25. The Fura 2 K_d was 168 ± 6.5 nM (n = 4), while that for Quin 2 was 110 ± 15 nM (n = 3). The lower K_d observed for Fura 2 in these experiments as compared to previously published values [16] probably reflects both differences in the ionic strength and the temperature used. The Quin 2 K_d is in good agreement with the previously reported K_d for Quin 2 at 20°C [31].

To quantitate cellular dye content, known amounts of Fura 2 or Quin 2 were added directly to the sample after recording F_4. Content was calculated after correcting the cellular calcium-sensitive fluorescence for calcium-insensitive fluorescence and expressed as nmol of dye/mg cell protein. Cell protein was determined by the method of Bradford [7] using BSA as a standard.

Oxygen Consumption

Cellular oxygen consumption (QO_2) for control and dye loaded suspensions were determined in a sealed temperature controlled chamber at 25°C with an oxygen electrode and amplifier (Instech).

Cellular Nucleotides

Nucleotides were extracted in $HClO_4$, prefrozen in liquid nitrogen and quantitated by high performance liquid chromatography [20]. Cellular nucleotides were calculated as total minus extracellular (generally <2% of the total) and normalized per mg cell protein.

Cellular Ion Content

Ca content was determined after washing 1 ml of suspension in 10 ml of Ba^{2+} substituted Ca^{2+} free medium containing (in mM) 102 NaCl, 3.5 KCl, 3 HEPES, 1 $MgCl_2$, 0.9 $BaCl_2$, pH 7.4 at 4°C. The resulting pellet was resuspended in Ba buffer and centrifuged through a 2 : 1 mixture of dibutyl/dioctyl phthalate. Na and K contents were determined after washing suspensions in *n*-methyl-D-glucamine substituted buffer as described for Ca content. Ions were quantitated in perchloric acid extracts by atomic absorption and normalized per mg cell protein.

Statistics

All group data are expressed as mean ± SD. Differences between groups were considered significant at $P < 0.05$, using the paired Student's *t* test.

Results

Initial studies examining control values for Ca_f using Fura 2 or Quin 2 indicated that the two dyes did not give identical values despite their similar K_d's at 25°C and the use of identical protocols to determine

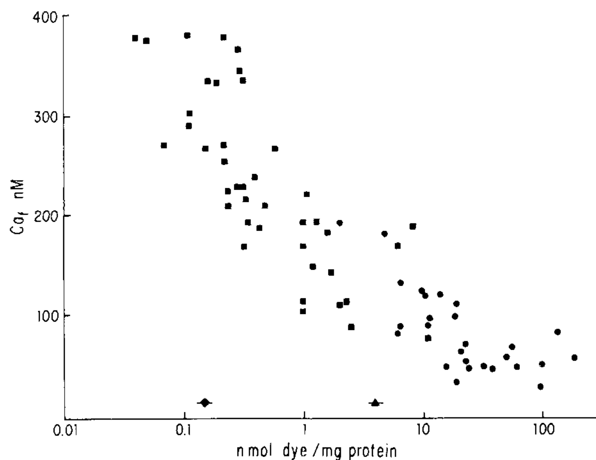

Fig. 1. Relationship between measured Ca_f and log cell dye content for Fura 2 (■) and Quin 2 (●) loaded cells. Cells were incubated at 25°C for 30 min prior to Ca_f determination. The autofluorescence of nondye loaded cells was also measured and converted to Fura 2 (♦) or Quin 2 (▲) equivalent fluorescence values per mg cell protein, and these values are plotted along the *x* axis

Ca_f[1]. Fura 2 loaded cells provided an average control value for Ca_f of 234 ± 13 nM (n = 39), while the average value using Quin 2 was 86 ± 9.4 nM (n = 22). The Quin 2 value is in good agreement with previous studies of Ca_f in isolated toad bladder cells using similar methods [36]. To determine if the difference in Ca_f between Fura 2 and Quin 2 loaded cells was due to differential dye loading, cellular dye content was varied from 0.04 to 180 nmol/mg protein by altering the concentration of Fura 2 acetoxymethylester (AM) or Quin 2 AM incubated with cells as well as the incubation time. The combined results for these studies are shown in Fig. 1, which clearly illustrates an inverse relationship between cellular dye content and Ca_f. While previous investigators using Quin 2 have not observed an effect of cell dye content on Ca_f, the range of dye content examined has generally been less than an order of magnitude. In the present study, examination of Fig. 1 over a limited range of Quin 2 content does not readily identify a relationship between Quin 2 content and Ca_f. With the availability of Fura 2, however, the dye content range has been expanded to greater than four orders of magnitude in the present study, thereby revealing the effect of dye content on Ca_f. A similar inverse relationship has been observed in suspensions of renal cortical tubules from the rabbit [21] but not in cortical tubule suspensions from the rat (*unpublished observation*).

To determine the cellular mechanisms responsible for this relationship, several possibilities were considered. The first was that dye loading altered cellular viability and/or metabolism, as previously reported for lymphocytes [18] and red blood cells [38]. Cellular QO_2 and nucleotide content were determined in dye loaded cells to evaluate this possibility. As shown in Table 1, neither Fura 2 or Quin 2

Table 1. QO_2 (nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1})

	Basal	+Nystatin	+Ouabain	nmol dye/mg protein
Control	9.1 ± 1.6	13.5 ± 2.7	5.7 ± 1.3	—
+Fura 2 (n = 4)	7.8 ± 1.1	11.6 ± 1.2	5.4 ± 0.7	2.67 ± 1.2
Control	8.4 ± 1.4	12.6 ± 2.2	5.3 ± 1.1	—
+Quin 2 (n = 5)	9.1 ± 1.5	14.8 ± 2.9	5.4 ± 1.3	36.8 ± 17.3
	Nucleotide Content (nmol/mg protein)			
	ATP	ADP	ATP/ADP	nmol dye/mg protein
Control	11.4 ± 1.7	1.5 ± 0.5	7.6	—
+Fura 2 (n = 3)	13.2 ± 2.3	1.8 ± 0.3	7.3	2.93 ± 1.7
Control	8.9 ± 0.6	1.2 ± 0.2	7.4	—
+Quin 2 (n = 4)	8.9 ± 1.7	1.2 ± 0.1	7.4	20.3 ± 10.8

Oxygen consumption was determined polarographically as described in Materials and Methods. After a 10-min basal recording, nystatin, 25 µg/ml, was added and the increase in QO_2 recorded for 5–10 min. Nystatin, a monovalent ionophore, increases intracellular Na^+, thereby increasing Na-K-ATPase activity and mitochondrial respiration [17]. Following nystatin, 1 mM ouabain was added to inhibit sodium pump activity and measure Na^+ independent QO_2. Nucleotides were measured as described in Materials and Methods.

[1] In preliminary studies, the ratio method for Ca_f determination using Fura 2 [16] was attempted and found to be unsatisfactory in isolated toad bladder cells. In examining the excitation spectra from dye-loaded cells it was observed that, even following extensive washing and autofluorescence correction, a residual Ca-insensitive signal, similar to the Fura 2 AM spectrum, was present. The residual signal substantially increased the observed 380 nM fluorescence, thereby reducing the calculated 350:380 ratio and seriously underestimating Ca_f when compared with values obtained using fractional occupancy calculations and 350 nM excitation. Recent studies by Scanlon, Williams and Fay [33] identified a contaminating signal from Fura 2 loaded cells, which could account for the changes in excitation spectra observed in these cells. A similar contaminant has been reported in isolated sarcoplasmic reticulum preparations [19].

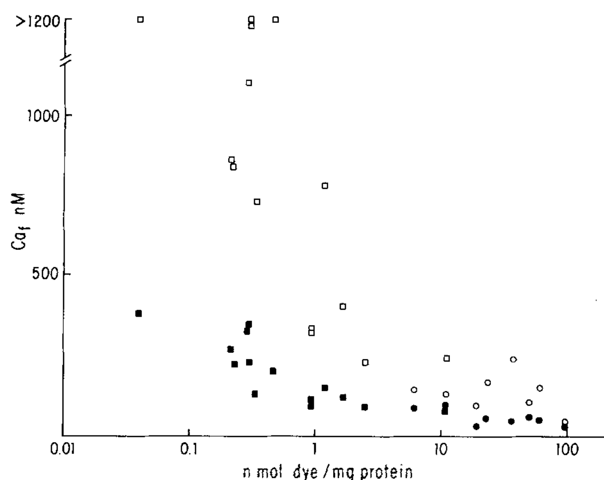

Fig. 2. Effect of 10 μM Br-A23187 addition on Ca_f. Both ionophore treated (open symbols) and paired controls (filled symbols) are shown for Fura 2 (□, ■) and Quin 2 (○, ●) loaded cells. Values for Ca_f were determined 5 min after addition of ionophore or vehicle

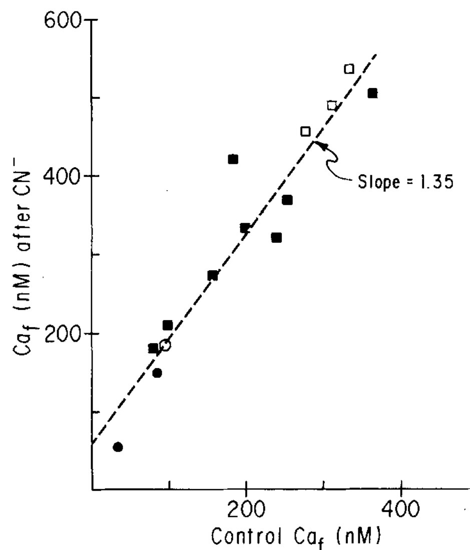

Fig. 3. The effect of 300 μM CN^- on control Ca_f and Ca_f in the presence of 10 μM Br-A23187. Control Ca_f (●, ■) and Ca_f in the presence of ionophore (○, □) are plotted along the x axis, while Ca_f in the presence of CN^- or CN^- plus ionophore are plotted along the y axis. Regression analysis indicated a correlation of 0.95 with a slope of 1.35 (n = 14)

altered basal QO_2 or cellular nucleotide levels and no differences in the QO_2 responses to nystatin or ouabain were observed. The possibility that trace amounts of Zn^{2+} may have artificially increased the Fura 2 signal [16] and reduced the Quin 2 signal [4, 18] was evaluated by incubating loaded cells with 45 μM of the cell permeant heavy metal chelator tetrakis[2-pyridylmethyl]ethylenediamine (TPEN), which has negligible affinities for Ca^{2+} or Mg^{2+} [4], for 5–10 min prior to determination of Ca_f. TPEN slightly increased mean Ca_f from 200 ± 45 nM to 227 ± 33 nM (n = 5) for Fura 2 and from 107 ± 23 nM to 120 ± 19 nM (n = 4) for Quin 2 loaded cells. Neither change was statistically significant, and no relationship between TPEN effect and dye content was noted.

The dependence of this relationship on external Ca was examined in two types of experiments. First, suspensions loaded with Fura 2 in the presence of 1 mM Ca were subsequently incubated for 30 min in a low (0.1 mM) Ca medium. This manuever reduced Ca_f from 192 ± 38 nM to 128 ± 22 nM, n = 4 $P < 0.05$, suggesting that reduced Ca entry may be partially responsible for the effect of increasing dye content on Ca_f. Second, the total Ca content of the cells was measured in the presence and absence of Quin 2 to determine whether the Ca bound to the dye comes mainly from internal sources or from net influx. Cells loaded with Quin 2 (18.0 ± 2.0 nmol Quin 2/mg protein; Ca_f = 79 ± 17 nM; n = 5) showed no significant difference in total cell calcium as compared to unloaded cells (17.7 ± 1.9 *vs.* 18.5 ± 2.9 nmol Ca/mg protein). This result suggests that most of the Ca bound to the dye comes from internal Ca redistribution, suggesting that the rate of Ca influx into these cells is low.

The inverse relationship between cell dye content and Ca_f was further characterized by examining the effects of Ca ionophores and CN^- on Ca_f. At low Fura 2 content (<0.5 nmol dye/mg protein), 10 μM Br-A23187 increased Ca_f to 700 nM or higher, reaching dye saturation in many cases (Fig. 2). At higher Fura 2 loading (>0.5 nmol dye/mg protein) and all levels of Quin 2 loading, Br-A23187 produced a subsaturating increase in Ca_f (Fig. 2). The latter response was achieved in about 1–2 min to a new value, which then remained constant through 5 min. At high Quin 2 content addition of Br-A23187 increased Ca_f from 57 ± 8.4 to 131 ± 24 nM (n = 8) and a delay in ionophore mediated increase in Ca_f was sometimes observed, consistent with studies in neutrophils [39]. Interestingly, the value for Ca_f in the presence of ionophore is close to the K_d of Quin 2 (115 nM), suggesting that the blunted ionophore effect reflects, in part, buffering of Ca_f by the dye [39]. Similar results were obtained using 30 μM ionomycin, except that signal saturation at low Fura 2 content was not observed. When lower concentrations of ionophores were tested, either transient or small stable increases in Ca_f were observed consistent with previous studies in a variety of cell types [18, 31, 36, 41]. When either ionophore was added to cells in the presence of 5 mM EGTA, no consis-

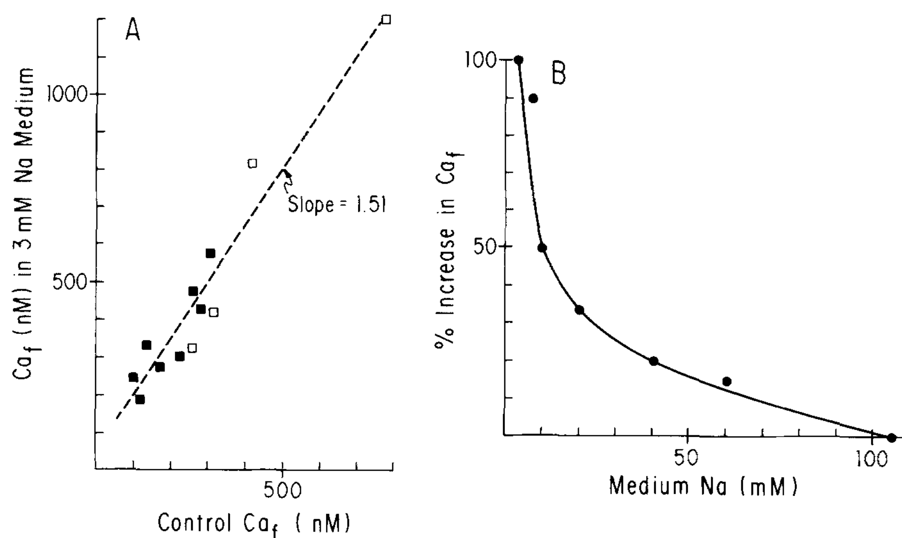

Fig. 4. Effect of reduced medium Na on Ca_f. Data is plotted as described in Fig. 3. (*A*) Cells were incubated with 3 mM Na, *n*-methyl-D-glucamine substituted medium, for 30 min at 25°C. Only Fura 2 loaded cells were examined. Control Ca_f (■) and Ca_f in the presence of Br-A23187 (□) are plotted along the *x* axis while Ca_f in Na substituted medium is plotted along the *y* axis. Regression analysis indicated a correlation of 0.88 with a slope of 1.51. (*B*) In three experiments the effect of varying medium Na on Ca_f was examined. Cells were loaded with Fura 2 and incubated as described for *A*. Control Ca_f = 280 ± 15 nM *vs*. 495 ± 43 nM with 3 mM Na medium

tent increase in Ca_f was observed, but rather a slow decline in Ca_f was noted. For both Br-A23187 and ionomycin, further addition of ionophore had no effect on the new Ca_f. However, digitonin addition always produced dye saturation in the absence of EGTA.

The effect of CN^- was tested to determine whether the limited increase in Ca_f after ionophore addition was due to a metabolically dependent process. Addition of 300 μM KCN increased Ca_f in both Fura 2 and Quin 2 loaded cells (Fig. 3). The increase in Ca_f was proportional to the control Ca_f over the range examined. When CN^- and Br-A23187 were added simultaneously, Ca_f was elevated to a higher level than observed with ionophore alone. Again, the increase in Ca_f with CN plus ionophore was proportional to the Ca_f observed with ionophore alone. This result suggests that the limited ionophoretic effect was not due to quenching of intracellular dye but to a combination of Ca buffering and a metabolically dependent Ca transport process.

To determine the role of Na-Ca exchange in the regulation of Ca_f the effects of medium sodium substitution (with *n*-methyl-D-glucamine) and ouabain on Ca_f were examined. Following a 30-min incubation in medium containing 3 mM Na, Ca_f was higher than in the presence of normal Na (Fig. 4*A*). As observed in the presence of CN^- the increase in Ca_f was proportional to the Ca_f in normal Na medium. In three studies the effect of varying medium Na on Ca_f was examined (Fig. 4*B*). The apparent K_m for the effect of medium sodium on Ca_f was 10–20 mM. Previous studies in toad bladder cells [36, 47], cultured epithelial cells [6, 35], rabbit proximal tubules [26] and *Necturus* proximal tubule [24] have also reported an increase in Ca_f following Na substitution. While increased Ca_f has been interpreted as evidence for Na-Ca exchange mediated Ca efflux, the use of nonpolarized cell suspensions in the present study makes this conclusion tenuous. For example, a reduction in medium Na may alter both Na-Ca exchange activity and mucosal Na channel activity, thereby altering intracellular sodium concentration. Previous studies have shown that reduction of medium Na reduces cell Na content [24, 28], and preliminary studies in this preparation have shown a greater than 50% reduction in cell Na and a slight decline in K content (*unpublished observations*).

To clarify the role for Na-Ca exchange in the regulation of Ca_f, the effect of ouabain on Ca_f was examined. Ouabain increases intracellular Na, thereby reducing the Na gradient for Na-Ca exchange. When low Fura 2 content suspensions (0.73 ± 0.25 nmol Fura 2/mg protein, $n = 8$) were preincubated with 1 mM ouabain for 30 min no significant change in Ca_f was observed (232 ± 36 nM *vs*. 221 ± 39 nM). Short-term (<5 min) ouabain treatment had no effect on Ca_f and ouabain treatment of Quin 2 loaded suspensions also had no effect on Ca_f (*data not shown*). When the concentration of oua-

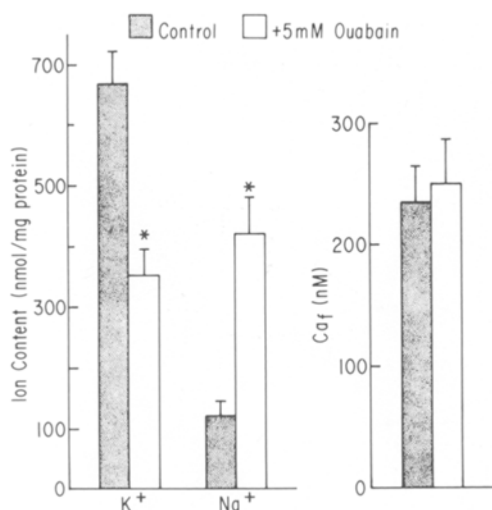

Fig. 5. Effect of 5 mM ouabain for 60 min on cell ion content and Ca_f ($n = 5$). In paired non-Fura 2 loaded cells ouabain reduced K content from 697 ± 48 to 362 ± 35 nmol/mg protein and increased sodium content from 137 ± 27 to 452 ± 52 nmol/mg protein

bain was increased to 5 mM and the incubation time extended to 60 min, no increase in Ca_f was observed despite a clear increase in cell Na content (Fig. 5). No change in Na + K content was observed (788 ± 65 *vs.* 773 ± 82 nmol/mg protein), suggesting that ouabain did not alter cell volume. The changes in cell K and Na content in the presence of ouabain were identical in Fura 2 loaded and nonloaded cells. However, in the presence of ouabain, addition of Br-A23187 or CN^- produced a greater increase in Ca_f than was observed in paired nontreated cells (Table 2). This difference in Ca_f observed with Br-A23187 and CN^- addition provides some evidence for the presence of Na-Ca exchange (*see* Discussion).

Table 2.

	Control	+5 mM Ouabain
+Br-A23187 ($n = 5$)	589 ± 55	796 ± 67*
+CN^- ($n = 4$)	325 ± 20	451 ± 57*

Effect of 10 μM Br-A23187 and 300 μM KCN on Ca_f in control and ouabain pretreated Fura 2 loaded cells. Cells were incubated for 1 hr in the presence or absence of 5 mM ouabain prior to addition of either 10 μM Br-A23187 or 300 μM CN^-. The effect of ionophore or CN^- was determined 2–3 min after addition. *$P <$ 0.05 compared to control.

Discussion

The present study was undertaken to compare the use of Fura 2 and Quin 2 for measurement of Ca_f in isolated toad bladder cells and examine the role of Na-Ca exchange in the regulation of Ca_f. The combined results of basal measurements of Ca_f clearly demonstrate an inverse relationship between cellular dye content and Ca_f. The mean value of Ca_f for Quin 2 loaded cells is in good agreement with previously reported values for toad bladder cells in which extracellular dye fluorescence was corrected [36] but lower than noncorrected values [9, 47]. At higher Quin 2 loading (50–180 nmol/mg protein), an apparent minimum value for Ca_f of 58 ± 8.4 nM ($n =$ 8) was observed, a value consistent with Ca_f measured in Quin 2 loaded toad bladder cells incubated in medium containing 0.1 mM Ca [36]. Conversely, at low Fura 2 loading (0.05–0.2 nmol/mg protein), no clear plateau value for Ca_f was observed. In light of the dye-dependent effect on basal Ca_f, it was anticipated that at low dye content the observed Ca_f would reach a constant value (due to minimal dye-Ca_f interaction), thereby indicating the true resting Ca_f. However, lower levels of Fura 2 content were not reliably measured due to the progressive dominance of cellular autofluorescence in the recordings. Although a clear plateau for Ca_f was not observed, the present data indicates a minimal value for resting Ca_f between 250–350 nM. This estimate is in good agreement with previously reported values for Ca_f in amphibian epithelial cells using Ca^{2+} microelectrodes [23, 24].

In an attempt to explain this inverse relationship, various possibilities were explored. This behavior does not appear to be due to dye-induced alterations in cell viability based on the observation that basal, nystatin-stimulated, and ouabain-inhibitable QO_2 are not altered by dye loading. The latter two variables are sensitive indicators for early changes in cell viability [34]. Additionally, dye loading does not adversely affect cellular ATP or ADP content, in contrast to previous studies [38, 41]. The difference between the Fura 2 and Quin 2 values for Ca_f does not appear to be related to a trace heavy metal effect [4, 18] in that TPEN does not significantly alter the observed Ca_f. Another possibility could be intracellular dye compartmentation, since recent studies in toad smooth muscle cells have shown that Fura 2 distributes into both the nuclear and sarcoplasmic reticulum, as well as cytosol, and

that these subcellular compartments have higher values for Ca_f than is observed in the cytosol [46]. In smooth muscle cells the concentration of Fura 2 in subcellular compartments tended to increase with larger dye loading and therefore would cause an overestimation of Ca_f using macroscopic techniques at high dye content. However, this type of phenomenon would not account for the present observation that Ca_f decreases with increasing dye content in toad bladder cells. The question of dye microcompartmentalization in toad bladder cells awaits more detailed analysis.

The data obtained with Ca ionophores suggests that the inverse relationship may be due, in part, to a dye-dependent buffering of Ca_f. Numerous investigators have suggested a Quin 2 dependent buffering action based on limited effects of ionophores, altered cellular responses to stimulate in the presence of Quin 2 [14, 22, 42, 45] and an effect of high *vs.* low dye content on basal Ca_f [36, 45]. However, buffering alone does not appear to account for the present data taken as a whole. Rather a combination of buffering and an alteration in cell Ca handling is suggested by the present data. A dye-dependent alteration of Ca handling is implicated by the following observations. First, Ca_f is constant for up to 2 hr after dye loading (longer time points have not been examined), suggesting that Ca_f is held at a new set point rather than transiently buffered to low levels as observed in hepatocytes [29]. Second, the effect of increasing dye content on Ca_f can be mimicked by incubating suspensions in a reduced Ca medium (*see* Results and Ref. 36), suggesting that a reduction in Ca permeability may account for the effect of dye content on basal Ca_f. Third, consistent with the second point, total cell Ca does not significantly increase in Quin 2 loaded cells, in contrast to studies in lymphocytes [18], red blood cells [38], and hepatocytes [10]. At a dye content of 18 nmol Quin 2/mg protein and a Ca_f of 79 nM, the calculated Ca bound to the dye is 7.4 nmol/mg protein. Had the cells accumulated Ca during dye loading and pre-incubation to offset the increasing dye content, a 7.4 nmol/mg protein increase in total cell Ca should have been observed. The lack of significant increase in total Ca content with Quin 2 loading suggests that the Ca bound to the dye is predominantly obtained from intracellular stores. This phenomenon may account for the inability to detect releasable Ca when ionophores are added to the absence of medium Ca. Fourth, the observation that CN^- increases both basal and ionophore-mediated Ca_f suggests that free calcium concentration is being maintained, in part, by energy-dependent processes even in the presence of the ionophore. Finally, the minimum value for Ca_f, 58 nM, observed with high Quin 2 is not the K_d for the dye, implying that this value of Ca_f is not just due to dye buffering[2].

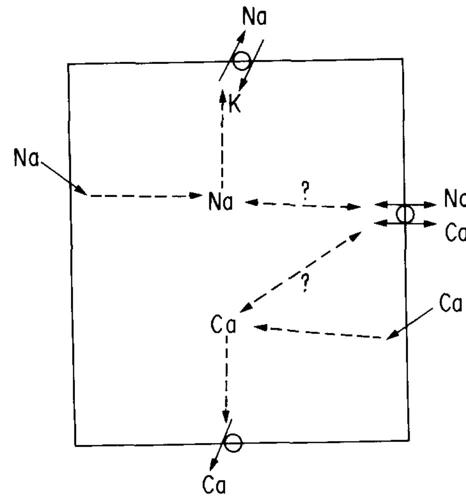

Fig. 6. Proposed pathways for cellular Ca_f regulation. *See* text for discussion

To explore the effect of dye content on basal Ca_f we considered a simple scheme for cell calcium handling shown in Fig. 6. For Ca influx, two pathways may be considered, including a Ca channel and/or Na-Ca exchange, whereas Ca efflux may occur via a Ca-ATPase and/or Na-Ca exchange. Na-Ca exchange may be considered as either a Ca influx or efflux pathway, depending on the relationship between plasma membrane Na and Ca gradients. While Na-Ca exchange has classically been predicted to operate as a Ca efflux pathway [43], recent studies have suggested that in epithelial cells Na-Ca exchange-mediated Ca influx may occur under normal cell conditions [35]. Using this scheme, the present observation that increasing dye content results in a sustained reduction in Ca_f may be attributed either to a reduction in Ca influx or an

[2] If the reduction in Ca_f was simply due to the introduction of a strong Ca buffer to a cell with inherently low Ca permeability, we would predict that Ca_f would plateau at the K_d for the dye, 168 nM for Fura 2 and 115 nM for Quin 2 loaded suspensions, respectively, as the increasing dye content becomes the dominant Ca buffer [39]. By analogy, addition of a strong basic buffer (Tris base) to an acetate buffered solution progressively increases pH. When Tris becomes the dominant buffer the solution pH will plateau at ≈8.2 (the pK for Tris). While pH will eventually become more alkaline as Tris base is added in excess, a clear plateau precedes alkalizination beyond the pK for the buffer.

increase in Ca efflux such that a new steady state is achieved.

Most of the results obtained in this communication suggest that a reduction in Ca influx occurred. First, based on the observation that Ca_f is stable for 2 hr after dye loading and total Ca content does not increase in proportion to dye content, it would appear that a dye-dependent reduction in Ca influx occurs such that Ca entry is reduced. Second, using low Fura 2 content suspensions, incubation in a reduced Ca medium decreases Ca_f, demonstrating that a reduction in Ca entry mimics the effect of high Quin 2 content on Ca_f. Interestingly, the apparent minimum value for Ca_f (58 nM) observed with high dye loading is in good agreement with the minimum value (54 nM) previously reported in Quin 2 loaded toad bladder cells incubated in reduced Ca medium [36]. The latter value was clearly due to a reduced Ca influx since readdition of Ca^{2+} to the medium progressively increased Ca_f to 89 nM. Third, while an increase in Ca efflux could, in theory, account for the effect of dye content on basal Ca_f (Fig. 1), the relationship observed in Fig. 3 would not be anticipated. For example, if a primary increase in Ca efflux accounted for the reduced basal Ca_f, it would be predicted that addition of CN^- to high dye content suspensions (low basal Ca_f) would have a greater effect on Ca_f than the same treatment in low dye content (high basal Ca_f) suspensions (assuming equitable reduction of ATP in both cases). These considerations taken together suggest that increasing dye content primarily reduces Ca influx. However, a simultaneous increase in Ca efflux, at high dye content, cannot be excluded by the present data and may in fact contribute to the limited effect of Br-A23187 at high Quin 2 content (Fig. 2). Further studies are needed to evaluate whether a simultaneous increase in Ca efflux contributes to the reduction in basal Ca_f.

While it is not clear by which mechanism the increase in dye content causes a reduction in Ca influx, one possibility is that Ca entry via Na-Ca exchange is reduced. Previous studies in squid axon have shown that reduction of cytosolic Ca, using either Quin 2 [1] or EGTA [13], paradoxically reduces Ca entry in exchange for cytosolic Na. It is important to note that in these studies reduction of cytosolic Ca did not alter Ca efflux in exchange for medium Na. Alternatively, a dye-dependent reduction of Ca_f may inactivate a Ca channel, also resulting in a sustained reduction of Ca_f.

To test for the presence of Na-Ca exchange activity the effect of increased intracellular Na, through ouabain treatment, and the effect of medium Na substitution were examined. Ouabain treatment alone did not elevate Ca_f despite a clear increase in intracellular Na content. Previous studies in cultured renal cells and proximal tubules have reported either an increase [6, 24] or no change [21, 26, 32, 35] in Ca_f with ouabain. In the present study ouabain inhibited QO_2 and altered cell ion content to the same extent in Fura 2 loaded and nonloaded cells, demonstrating that Fura 2 did not interfere with ouabain inhibition of the sodium pump. The lack of effect of ouabain on basal Ca_f suggests that Na-Ca exchange does not significantly contribute to Ca_f regulation under basal conditions. On the other hand, the facilitated effect of Br-A23187 and CN^- in ouabain pretreated cells may indicate that Na-Ca exchange may become an important Ca efflux pathway only at elevated Ca_f.

In contrast to the lack of effect of ouabain on Ca_f, medium Na substitution resulted in a sustained elevation of Ca_f. Previous studies mainly in non-epithelial [27, 28, 30] cells have shown an increase in Ca_f with either ouabain or medium Na substitution, consistent with Na-Ca exchange-mediated Ca efflux. The disparity found in the present experiments between the effect of medium Na substitution and ouabain suggests that the effect of Na substitution may not be mediated via Na-Ca exchange [26]. Medium sodium substitution alters a number of cell variables, including reducing total cation content and altering external plasma membrane ion binding, which are not affected by ouabain. These additional nonspecific effects of medium Na substitution may elicit changes in Ca_f by mechanisms unrelated to Na-Ca exchange. Alternatively, recent studies have suggested that Na-Ca exchange may not be a symmetrical transport system [1, 13]. In particular, extracellular Na competes for the extracellular Ca binding site and may reduce Ca influx via Na-Ca exchange [13]. A reduction in medium Na concentration would then facilitate Ca influx via Na-Ca exchange by (i) providing a more favorable gradient for Na exit [35] and (ii) removing Na inhibition of extracellular Ca binding. If Ca influx is mediated, in part, by Na-Ca exchange and if a reduction in medium Na facilitates Ca entry, then an increase in Ca_f similar to the effect of Ca ionophores would be observed. By contrast, the increase in intracellular Na with ouabain treatment would not alter Na competition for extracellular Ca binding and, therefore, may not perturb Ca influx sufficiently to produce a measurable increase in Ca_f. Further studies are needed, however, to clarify the role of Na-Ca exchange as a Ca influx pathway.

With regard to the role of Ca_f in the regulation of Na transport, it is interesting to note that neither Fura 2 nor Quin 2 altered basal QO_2 or the response to nystatin and ouabain (Table 1). Nystatin stimulation of QO_2 reflects the effect of increased Na entry,

while ouabain inhibition of QO_2 represents the minimum, zero transport, QO_2. Within this range of Na-dependent QO_2, changes in Ca_f from control values (presumably 250–350 nM as inferred from Fig. 1) to 203 nM (Fura 2) or 91 nM (Quin 2) had no effect on basal QO_2. This observation, although indirect, suggests that profound changes in Na transport do not occur when Ca_f is reduced over the range examined in the present study.

The current availability of Fura 2 and Quin 2 to measure Ca_f has extended the range of experimentally obtained dye contents and revealed a strong dye-dependent effect on Ca_f in isolated toad bladder cells. The mechanism by which dye content alters Ca_f is not understood in detail but appears to be related to an alteration in cell Ca_f set point and buffering action of the dyes. At low Fura 2 content, minimal alterations in Ca steady state were observed, suggesting that at this level of dye loading useful information regarding regulation of Ca_f can be obtained. In this context, the absence of significant changes in Ca_f following ouabain treatment suggests a minimal role for Na-Ca exchange as a Ca efflux pathway. Alternatively, the strong dye-dependent effect on Ca_f suggests that Fura 2 and Quin 2 may be useful agents by which Ca_f can be "clamped" and the effects of physiological stimuli examined at documented values for Ca_f. Further studies in intact epithelial preparation are needed, however, to evaluate whether the effect of dye content on Ca handling is unique to isolated cells.

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