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Fura-2 transport in toad urinary bladder epithelium: effects of antidiuretic hormone, colchicine and osmotic gradients

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Fluorescence is transferred across the toad urinary bladder when fura-2/AM is added to the mucosal or serosal sides of the epithelium. It was now observed that: (1) Oxytocin (20 nM, serosal) increased fluorescence transfer from the mucosal to the serosal but not from the serosal to the mucosal baths. The ratio between the fluorescence intensities recorded with excitation wavelengths of 340 and 380 nm indicates that the calcium sensitive probe (free fura-2) was transferred to the serosal but not to the mucosal compartment by an oxytocin sensitive transport. (2) Preincubation with probenecid did not change fluorescence transfer in basal conditions but significantly reduced the oxytocin induced increase in free fura-2 transport. (3) Fluorescence accumulation inside the tissue was strongly reduced by oxytocin, but only when fura-2/AM was added to the mucosal side. (4) An osmotic gradient, in the presence of oxytocin, further increased the transfer of fluorescence at 380 nm but not at 340 nm. This indicated that the transfer of a calcium-insensitive fraction was being stimulated. (5) Preincubation with colchicine strongly inhibited fluorescence transfer across the tissue, at both 340 and 380 nm (the 340/380 ratio did not change). (6) Tissue accumulation was increased by colchicine. (7) Vanadate did not inhibit fura-2 transfer in the toad urinary bladder. We conclude that intracellularly-generated free fura-2 is only transported across the basolateral border, and that this transfer is stimulated by ADH. The calcium-insensitive fraction is transferred by a temperature-dependent process, sensitive to an osmotic gradient and colchicine.

Introduction

The fluorescent dye fura-2 has been employed to measure the cytoplasmic concentration of free ionized Ca^{2+} in a variety of cells [7,11,27]. The method generally employed consists in the exposure of the tested cells or tissue to a membrane-permeable derivative, the acetomethyl ester fura-2/AM. This lipophilic molecule enters the cell whereupon it is cleaved by cytosolic estearases liberating free fura-2, which is the calcium-sensitive molecule. Nevertheless, in certain cells free fura-2 tends to leak out to the extracellular fluid [18,27]. This phenomenon seems to be mediated, at least in certain cells, by an organic ion transport system and blocked by the inhibitors probenecid and sulfipyrazine [9].

Previously we reported that fura-2 is transferred across the toad urinary bladder by a temperature-dependent process [22]. Furthermore, free fura-2 is transported from the intracellular to the serosal compartment, across the basolateral membrane, but not into the mucosal bath. Because of this we proposed that fura-2, currently employed as a calcium probe, could be a useful tool for studying the transepithelial transport of polyclic compounds.

The aim of the present work was to gain further insight into the mechanisms underlying the transports of free fura-2 and fura-2/AM. Together with the actions of probenecid and vanadate, the effects of antidiuretic hormone (ADH), colchicine and osmotic gradients, all agents that in different ways modify transepithelial permeability in the toad urinary bladder, were tested.

Materials and Methods

Urinary bladders from pithed toads (*Bufo arenarum*) were perfused 'in situ' via the ventricular cavity

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until free of blood. The solution employed (Standard medium) contained (mM): 112 NaCl; 5 KCl; 1 $CaCl_2$; 2.5 $NaHCO_3$ (pH 8.1 when bubbled with air). The bladders were removed, everted when necessary, divided into two paired sacs and mounted in polypropylene holders, as previously described [22]. The sacs were filled and placed in a 3 ml quartz cuvette containing the standard medium. External and internal volumes were appropriated for obtaining a bladder sac of cylindrical shape (1200 and 500 μl, respectively). The quartz cuvettes were then placed on the stage of a Jasco 770 computed spectrofluorometer. To obtain the excitation spectra, the samples were excited between 310 and 500 nm, with a data interval of 0.5 nm, a screening speed of 100 nm/min, an excitation slit of 3 nm and an emission slit of 10 nm. Emission was recorded at 510 nm. Five consecutive cycles were automatically run and averaged in each case.

The spontaneous fluorescence of the tissue was initially screened in all experiments and taken as a reference value. Neither free fura-2 nor fura-2/AM had any detectable emission at 440 nm and this wavelength was therefore chosen to control eventual autofluorescence variations during the measurement periods.

Fura-2/AM (Sigma or Molecular Probes) was prepared in dry DMSO inmediately before use and added (5 μM) to the solution outside the sac, which represented either the mucosal (everted bladders) or the serosal bath (non-everted bladders).

After 120 min the solution inside the sac was removed and transferred to a microcuvette (volume 250 μl) for measurement of the fluorescence appearing in the medium. Both sides of the sac were then washed three times and the tissue fluorescence measured.

All experiments were made at 20°C. Two pieces of the same bladder were simultaneously tested in most cases. Osmotic gradients were created by reducing the NaCl concentration in the mucosal bath (5.6 mM). Fluorescence data were transferred to a PC-compatible computer via a RS-232 interface and saved for subsequent analysis. Oxytocin (Sintocinon, Sandoz) was used as an ADH analogue [3]. Colchicine, vanadate and probenecid were purchased from Sigma. Fura-2 is used through the text as a generic expression covering both free fura-2 and fura-2/AM.

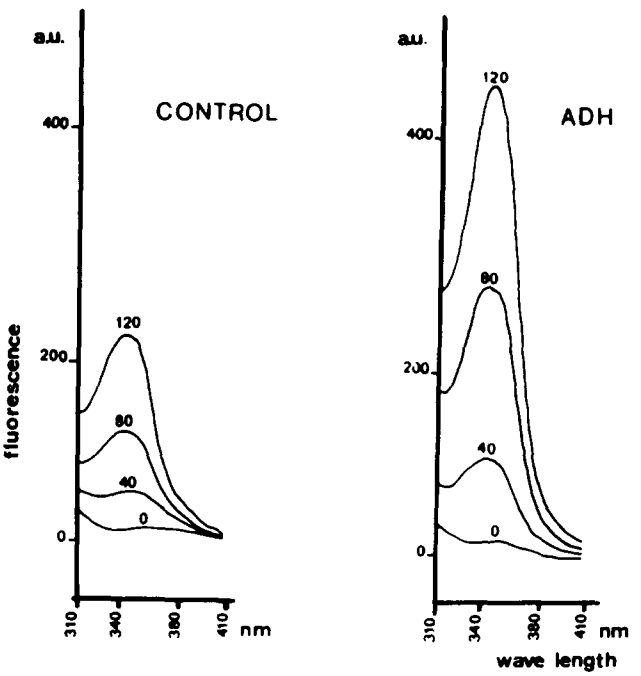

Fig. 1. Effect of ADH on the transfer of fluorescence across the toad urinary bladder. Excitation spectra of the serosal bath (emission recorded at 510 nm). Numbers on curves indicate minutes after fura-2/AM addition to the mucosal bath. Control and ADH treated (oxytocin, 20 nM, serosal) were paired hemibladders.

Results

Effect of oxytocin probenecid and vanadate on fluorescence transfer

Fluorescence is transferred across the urinary bladder when fura-2/AM is added to either the mucosal or the serosal baths.

Mucosa to serosa transfer. Fig. 1 shows the progressive appearance of fluorescence in the serosal bath when fura-2/AM (5 μM) was added to the apical compartment. It can be seen that oxytocin (ADH; 20

TABLE I

Effects of ADH (oxytocin, 20 nM) on fluorescence transfer across the toad urinary bladder

Fura-2/AM (5 μM) was added either to the mucosal (muc) or to the serosal (ser) bath 120 min before measurements. Intensity of emission at 510 nm (in arbitrary units) was measured in samples from the contralateral side. Data presented are for excitation wavelengths of 340 and 380 nm. *R*: 340/380. *n* = number of paired hemibladders. n.s., non significant.

Condition	Fura-2/AM,muc				Fura-2/AM,ser			
	n	340	380	*R*	*n*	340	380	*R*
Control	12	174±19	65±11	3.1±0.4	6	153±28	120±25	2.0±0.5
Oxytocin	12	250±25	69± 9	4.2±0.6	6	101±13	60± 9	2.3±0.4
Diff		76±21	4± 8	1.1±0.3		−53±14	−60±15	0.2±0.4
P		< 0.01	n.s.	< 0.05		< 0.02	< 0.02	n.s.

nM, serosal) increased fluorescence transfer (two hemibladders were compared). The ratio between the fluorescence intensities recorded with excitation wavelengths of 340 and 380 nm indicates, at a constant Ca^{2+} concentration (2 mequiv./l in this case), the proportions of calcium sensitive and calcium non sensitive probes present in the tested medium [22]. Table I shows that the effect of ADH on the mucosal to serosal transfer was observed at 340 nm but not at 380 nm. Consequently, the 340/380 ratio was significantly higher under hormonal stimulation, indicating a higher proportion of a calcium sensitive probe in the transferred mixture.

Addition of EGTA to a free fura-2 and fura-2/AM mixture shifts the 340/380 ratio towards lower values [22]. The magnitude of this shift gives information concerning the presence of a Ca^{2+}-sensitive probe (probably free fura-2) in the medium. Fig. 2 shows the effects of EGTA (4 mM) on the 340/380 ratio measured on the serosal and the mucosal sides, 120 min after fura-2 addition (5 μM) to the contralateral bath. It can be observed (Fig. 2B) that the calcium-sensitive fraction was larger, in the serosal side, after ADH action (Control: no-EGTA 3.31 ± 0.12; EGTA 2.26 ± 0.07; Diff 1.05 ± 0.10. After ADH: no-EGTA 4.28 ± 0.20; EGTA 2.46 ± 0.11; Diff 1.82 ± 0.14, $n = 8$). These results reinforce the view that oxytocin increases free fura-2 transfer into the serosal solution.

Effects of probenecid and vanadate. It has been previously reported that probenecid inhibits free fura-2 transfer in mammalian macrophages [9]. Table II shows fluorescence transfer, after fura-2/AM addition to the mucosal solution, in control condition or when probenecid (1 mM) was previously added to the serosal bath (5 min preincubation time). Two series were run, one with non-stimulated bladders and the other after oxytocin stimulation. Probenecid did not affect fluorescence transfer in non-stimulated bladders. Nevertheless, it reduced the increase induced by oxytocyn at 340 nm.

Vanadate, that inhibits the extrusion of a fluorescent probe in *Lactococcus lactis* [19], did not modify

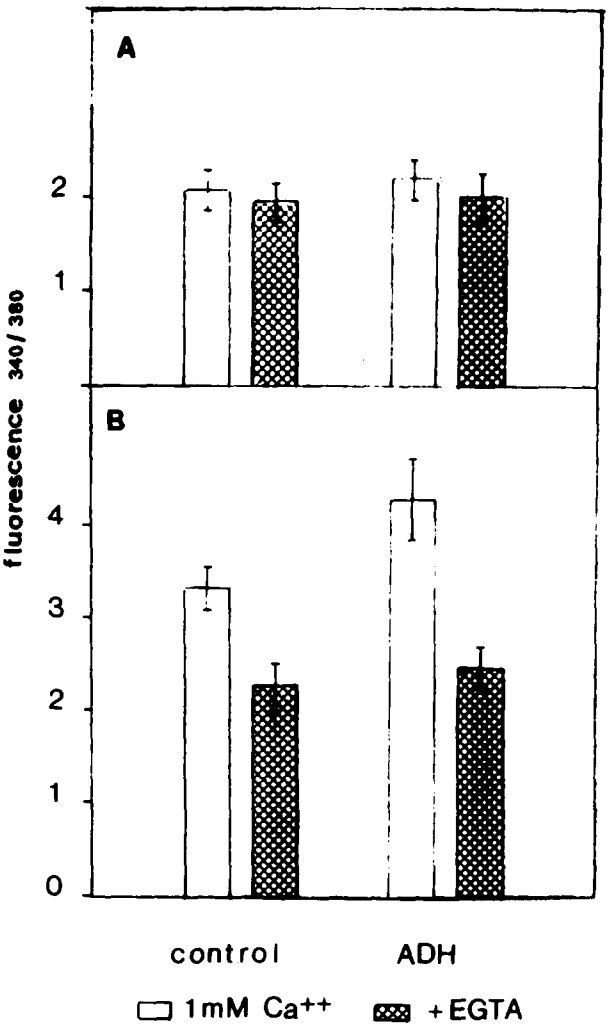

Fig. 2. Effect of EGTA (4 mM) on the 340 nm/380 nm fluorescence ratio measured in the mucosal (A) or serosal (B) bath. Control and ADH (oxytocin)-treated hemibladders are compared. Fura-2/AM was added to the contralateral side 120 min before.

fura-2 transfer across the toad urinary bladder (Fluorescence transfer in arbitrary units: Control – vanadate difference, 340 nm = 41 ± 47; 380 nm = 17 ± 18 ($n = 5$)).

Serosa to mucosa transfer. Oxytocin reduced the fluorescence transfer from the serosal to the mucosal

TABLE II

Effects of preincubation with probenecid (1 mM) on fluorescence transfer across the toad urinary bladder

Fura-2/AM (5 μM) was added to the mucosal bath 120 min before measurements. Effects on non-stimulated and oxytocin stimulated (ADH, 20 nM) bladders are compared. Data were obtained as indicated in Table I. n = number of paired hemibladders. n.s., non significant.

Condition	No ADH				ADH			
	n	340	380	R	n	340	380	R
Control	5	93 ± 38	44 ± 17	2.1 ± 0.6	6	480 ± 94	105 ± 15	4.6 ± 0.7
Probenecid	5	103 ± 46	53 ± 14	1.9 ± 0.5	6	360 ± 60	95 ± 18	3.8 ± 0.4
Diff		16 ± 22	25 ± 11			120 ± 41	10 ± 16	
P		n.s.	n.s.			< 0.05	n.s.	

side (Table I), but the 340/380 ratio did not change. Furthermore, no EGTA sensitive fraction was observed in the apical compartment before or after ADH action (Fig. 2,A) These results confirm that there is little, if any, transfer of free fura-2 to the mucosal compartment.

Effect of oxytocin on fluorescence accumulation inside the bladder tissue

Table III indicates that fluorescence accumulation inside the tissue was considerably reduced under oxytocin, but only when fura-2/AM was added to the mucosal side. In this situation the lipophilic probe only has free access to the epithelial cells. When fura-2/AM is added to the serosal compartment it can also enter the supporting components: muscle and vascular cells and conjunctive tissue. This situation explains the higher levels of fluorescence which probably mask the oxytocin action on the epithelial cells. The 340/380 ratios are not given in Table II because this parameter did not change in a predictable way for three reasons: The tissue complexity, the uneven distribution of free fura and fura-2/AM and the lack of Ca^{2+} concentration values.

Transepithelial osmotic gradients and ADH action

ADH induces, in the presence of an osmotic gradient, a net water movement in target tissues [13]. This water transfer is associated with cytoplasmic vacuolization and dilation of the intercellular spaces [10,23]. Table IV shows that the presence of an osmotic gradient increased the ADH-stimulated transfer of fluorescence from the mucosal to the serosal side at 380 nm but not at 340 nm. Consequently, the 340/380 ratio dropped to lower values. This indicates that free fura-2 transfer was not further increased by the presence of an osmotic gradient. On the other hand, the transfer of a calcium-insensitive fraction was clearly stimulated. Tissue accumulation under oxytocin action did not exist (Table III) or it was very low (Table IV). This situation did not change in the presence of an osmotic gradient.

TABLE III

Effect of ADH (oxytocin 20 nM) on the fluorescence accumulation inside the tissue

Intensity of emission in arbitrary units. Experimental conditions were as those described in Table I. n.s., non significant.

	Fura-2/AM,muc			Fura-2/AM,ser		
	n	340	380	*n*	340	380
Control	10	35±9	16±4	6	220±55	59±17
Oxytocin	10	2±6	3±5	6	172±23	55±12
Diff		−35±9	−18±6		−49±43	−4±12
P		< 0.01	< 0.02		n.s.	n.s.

TABLE IV

Effect of the presence of an osmotic gradient (mucosal side hypotonic) on oxytocin stimulated transfer and tissue accumulation

Fura-2/AM (5 μM) was added to the mucosal side. Other conditions as described in Table I ($n = 6$). n.s., non significant.

	Transfer			Accumulation	
	340	380	*R*	340	380
Oxytocin	238±35	60± 11	4.2±0.6	14±10	3± 7
Plus osm. gradient	374±94	165± 30	2.5±0.7	1±10	−6±18
Diff	135±82	105±129	−1.7±0.3	−14±10	−10±30
P	n.s.	< 0.05	< 0.01	n.s.	n.s.

Effect of colchicine on fura-2 handling in the toad urinary bladder

Incubation with colchicine disrupts the microtubule network in the toad urinary bladder [29], modifying considerably their permeability properties [24]. Fig. 3 and Table V clearly show that preincubation with

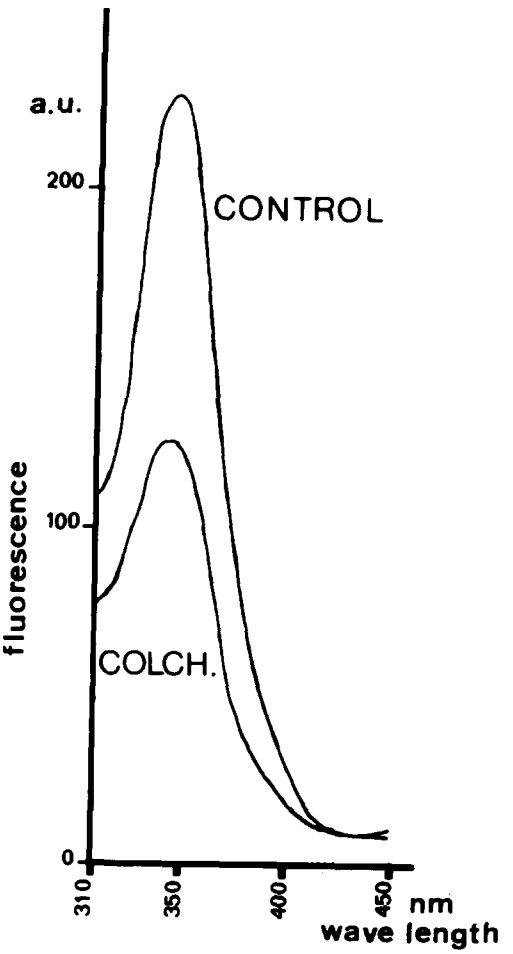

Fig. 3. Effects of colchicine (COLCH, 0.1 mM, serosal, 2 h preincubation time) on the transfer of fluorescence across the toad urinary bladder. Excitation spectra 2 h after addition of fura-2/AM to the mucosal side. Two hemibladders are compared.

TABLE V

Effect of colchicine (2 h pre-incubation period, 0.1 mM in the serosal bath) on fluorescence transfer and fluorescence accumulation inside the tissue

Fura-2/AM (5 μM) was added to the mucosal side 120 min before the measurements ($n = 6$). n.s., non significant.

	Transfer			Accumulation	
	340	380	*R*	340	380
Control	210±32	76±14	2.8±0.4	20± 9	16± 4
Colchicine	76±15	33± 8	2.3±0.3	320±59	180±30
Diff	−134±21	−43± 5	−0.4±0.3	+300±60	+164±32
P	< 0.01	< 0.05	n.s.	< 0.01	< 0.01

colchicine strongly inhibited fluorescence transfer across the tissue at both 340 and 380 nm (the 340/380 ratio did not change). As expected, EGTA addition reduced the 340/380 value in both control (−67 ± 7%) and colchicine treated-bladders (−52 ± 6%). These experiments were run in non oxytocin stimulated urinary bladders.

Tissue accumulation was greatly increased by colchicine at both 340 and 380 nm (Table V).

Discussion

Interest in the handling of macrocyclic compounds by cells has much increased in recent years. This could be one of several processes of importance in cell detoxification mechanisms and in the development of cell resistance to certain pharmacological agents [14]. The toad urinary bladder has been widely employed for testing water and solute transfers [3,13]. In the present study this epithelial barrier has been used to investigate the transfer of fura-2, a fluorescent polycyclic compound.

The main ADH-induced changes in target organs are cyclic AMP mediated and located at the apical surface of the cells [5,12]. They include: (1) An increased exo-endocytotic activity [17]; (2) an enlargement of the apical membrane area [20] and (3) the incorporation of aggregates, probably containing water channels, into the mucosal plasma membrane [4]. These processes are modulated by the intracellular Ca^{2+} concentration [21,30]. In a previous paper we reported that, in control conditions, a mixture of calcium sensitive and calcium non-sensitive derivatives appears in the serosal compartment 2 h after the addition of fura-2/AM (5 μM) to the mucosal bath [22]. The results presented here clearly show that oxytocin increased this fluorescence transfer across the toad urinary bladder at 340 nm but not at 380 nm, giving higher values for the 340/380 ratio. Together with the effects of EGTA these observations can be straightforwardly interpreted as an increase in free fura-2 transfer across the basolateral membrane. When fura-2/AM was added to the serosal compartment the fluorescence appearing in the mucosal bath had no calcium-sensitive component. This transfer even diminished after oxytocin action, with no change in the 340/380 ratio. Furthermore, no calcium sensitive fraction was detected in the apical compartment. We can conclude that free fura-2 is only transferred across the basolateral border, and that this transfer is stimulated by ADH action.

The presence of an osmotic gradient, in ADH-treated bladders, increased the transepithelial transfer of the calcium-insensitive fraction. It is possible that fura-2/AM, once in the cytoplasm, is subject to solvent drug. It is also possible that solute polarization phenomena, induced by the water flux, are at the origin of the observed increase in fluorescence transfer.

We have already shown that low temperature completely stops fluorescence transfer [22] and also reduces the ADH induced water movement [6]. Furthermore, disruption of the microtubule network inhibits the ADH-induced hydro-osmotic response [24,25] and we report here that colchicine strongly reduced the fluorescence transfer. We conclude that fura-2/AM is definitely not trans-epithelially transferred by a diffusive process. On the contrary, the vacuolar and microtubular structures must play a central role in the observed phenomena.

The results concerning fluorescence accumulation inside the tissue must be interpreted cautiously. Changes in fluorescence signals can be affected by various factors. Nevertheless the strong reduction in fluorescence accumulation observed in the presence of ADH is in agreement with an increase in the fura-2 exit across the basolateral membrane. In the same way, the increase in fluorescence accumulation under the action of colchicine is in accordance with an inhibition of fura-2 intracellular traffic while fura-2/AM entry across the mucosal border is probably not affected.

Di Virgilio et al. [9] have shown that secretion of fura-2 occurs, in mouse peritoneal macrophages, via organic anion transport systems sensitive to probenecid. We are now reporting that probenecid inhibited the oxytocin induced increase in fluorescence transfer at 340 nm. This observation can be interpreted as a direct inhibitory effect of probenecid on the free fura-2 transporter, located at the basolateral membrane. Nevertheless, it has been previously reported that probenecid inhibits the hydrosmotic response to oxytocin while potentiating the effect of cyclic AMP [26]. These results were attributed to a facilitated transfer of the nucleotide across the basolateral membrane. Then, a non-direct effect of probenecid can not be excluded.

It has been reported that bis(carboxymethyl) carboxyfluorescein (BCECF) is transported by an ATP-dependent mechanism in epithelial cells [1]. This system also shows membrane polarity [8] and can be

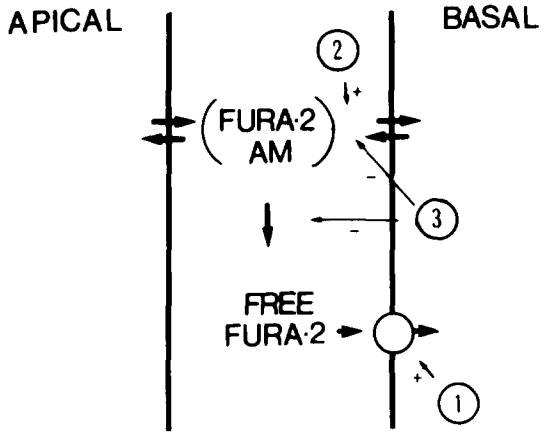

Fig. 4. Proposed model for fura-2 handling and transport in the toad urinary bladder. 1, ADH (oxytocin); 2, osmotic gradient (net water flux) and 3, colchicine.

inhibited by indomethacin but not by probenecid. BCECF extrusion is also catalysed by an ATP-driven extrusion system in *Lactococcus lactis* [19] that is inhibited by vanadate. It can be also mentioned that the polyamine transport by vacuolar membrane vesicles of *Saccharomyces cerevisiae* is mediated by a H^+-ATPase not sensitive to vanadate [16]. The here described fura-2 transport system(s) was (were) not sensitive to vanadate. Finally, reference should also be made to the fact that Fluo-3, also a Ca^{2+}-sensitive probe, is a possible substrate por an apical membrane transporter (P-glycoprotein) in epithelial cells [31].

Fura-2 and related molecules have been used to measure cytosolic Ca^{2+} concentration in this [15,30,33] and similar epithelial barriers. Our results show that fura-2 uptake, distribution and transfer across the toad urinary bladder is a complex process, as it is the case for other systems already studied [2,32]. The Ca^{2+}-insensitive component probably results, as previously reported in polymorphonuclear leukocytes [28] from incomplete deesterification of fura-2/AM by the cells.

Fig. 4 gives a proposed model for fura-2 handling in the toad urinary bladder. Once inside the epithelial cells fura-2 derivatives would be trapped in a vesicular compartment and transferred, by a temperature-dependent, colchicine-sensitive process, to the contralateral side. Part of this fura-2 is cleaved to free fura-2, which is taken up by an ADH-sensitive transport system, located in the laterobasal membrane. Future experiments will clarify the molecular mechanism underlying these transport mechanisms.

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