Evidence for intracellular sodium pumps in permeabilized *Xenopus laevis* oocytes

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Ouabain binding was studied in *Xenopus laevis* oocytes permeabilized by detergents. The behaviour of markers showed that 10 μM -digitonin selectively disrupts the plasma membrane. In the presence of ATP, oocytes permeabilized at 10 μM -digitonin bound no more ouabain molecules than were required to abolish active $^{86}\text{Rb}^+$ uptake in the intact cells. However, the ouabain binding capacity increased approx. 2-fold when inner membranes were disrupted by SDS or excess digitonin, as judged from the accompanying release of the lysosomal marker β -hexosaminidase. The results suggest that oocytes have a large internal pool of functional sodium pumps.

INTRODUCTION

The Na^+/K^+ -ATPase is a membrane-spanning enzyme, composed of the catalytic α -subunit and a glycosylated β -subunit of unknown function. During the course of their biosynthesis, the subunits are cotranslationally inserted into the membranes of the rough endoplasmic reticulum [1,2]. Newly synthesized α - and β -subunits assemble rapidly into stoichiometric complexes [1,3]. After 10 min, when the enzyme still resides in the endoplasmic reticulum [3], it is already competent to bind the cardiac glycoside ouabain [4]. The Na^+/K^+ -ATPase matures further on its passage through the Golgi apparatus [3,5] and arrives at the cell surface with a lag of about 30–90 min after its synthesis [3,4,6].

During the normal process of cellular turnover, Na^+/K^+ pumps are constantly delivered to and removed from the plasma membrane, suggesting that Na^+/K^+ pumps exist in the cell interior. As part of an ongoing study on the regulation of Na^+/K^+ -ATPase in *Xenopus* oocytes [7], we examined whether or not these cells have subcellular ouabain binding sites. Since ouabain binds to an ecto-domain of the phosphorylated α -subunit, it labels Na^+/K^+ pumps of the cell surface, but does not enter the cell by itself. To make inner membranes accessible for ouabain and ATP, we permeabilized oocytes with detergents. The plasma membrane was selectively disrupted by a low concentration of digitonin [8] and intracellular membranes by excess digitonin or by the nonspecific detergent SDS. Our results suggest that about half of the functional Na^+/K^+ pumps of prophase-arrested oocytes are located intracellularly.

EXPERIMENTAL

Chemicals

[^3H]Ouabain (15.6 Ci/mmol and 21 Ci/mmol), $^{36}\text{Cl}^-$ and $^{86}\text{Rb}^+$ were purchased from Amersham Buchler. Collagenase (0.6–0.8 units/mg) and digitonin were from Serva (Heidelberg, Germany) and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide was from Sigma Chemie

(München, Germany). Synthetic ATP was from Boehringer (Mannheim, Germany). All other chemicals (analytical grade) were purchased from Merck (Darmstadt, Germany).

Isolation and permeabilization of oocytes

Oocytes of adult *Xenopus laevis* (Xenopus Ltd., Surrey, England) were defolliculated by collagenase treatment as described [7]. The largest oocytes of a batch corresponding to stages V and VI according to Dumont [9] were manually selected under the microscope. For permeabilization and K^+ depletion, up to 40 cells per 10 ml of a NaCl/Tris medium (110 mM-NaCl/2 mM- MgCl_2 /1 mM-EGTA/10 mM-Tris, pH 7.4) were treated with 1–300 μM -digitonin. After 30 min at 5 °C, the permeabilizing medium was replaced by Na/Tris buffer free of digitonin, and the incubation was continued for an additional 30 min. According to stereomicroscopic examination, permeabilization did not cause visible damage to the cells.

Ouabain binding to permeabilized oocytes

Permeabilized oocytes were assayed for [^3H]ouabain binding at 25 °C in the Na/Tris medium (10 cells per 0.5 ml) containing 1 mM-ATP. For nonspecific binding, 1 mM unlabelled ouabain was included in the reaction mixture. At the desired time points, oocytes were removed and washed with Na/Tris medium. Unbound internal [^3H]ouabain was released by incubating the cells for an additional 1 h in the Na/Tris medium at 5 °C under slow shaking. Individual oocytes were dissolved separately in 5% SDS and counted in Quickszint 2000 (Zinsser, Frankfurt, Germany).

Ouabain binding and $^{86}\text{Rb}^+$ uptake in intact oocytes

Intact oocytes preincubated in K^+ -free oocyte Ringer solution (ORI: 90 mM-NaCl/1 mM- CaCl_2 /1 mM- MgCl_2 /10 mM-Hepes, pH 7.4) for 3–4 h were pulse-labelled with 0.2 μM -[^3H]ouabain for 5 min at 25 °C. Immediately after removing the unbound label by washing, the cells were reincubated in fresh K^+ -free ORI containing 1 mM- $^{86}\text{Rb}^+$

Abbreviations used: K_d , apparent dissociation constant; B_{max} , ouabain-binding capacity.

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(10 $\mu\text{Ci}/\text{ml}$), a K^+ analogue. Total and ouabain-insensitive uptakes of $^{86}\text{Rb}^+$ were determined in control cells preincubated in the absence and presence of 1 mM-ouabain respectively. After 10 min at ambient temperature, the oocytes were washed in ice-cold medium and prepared for liquid scintillation counting as above. The spillover of $^{86}\text{Rb}^+$ into the set ^3H -channel was less than 2%. Rb^+ uptake was linear for at least 20 min under control and ouabain-inhibited conditions. Active $^{86}\text{Rb}^+$ transport was calculated by subtracting ouabain-insensitive uptake. The number of ouabain molecules required for inhibition of active $^{86}\text{Rb}^+$ uptake (B_{max}) could be calculated by dividing the amount of ouabain bound (fmol) by the fraction of active $^{86}\text{Rb}^+$ transport blocked.

Determination of the release of internal markers

Oocytes were treated with digitonin as described above. At defined times, aliquots of the medium were withdrawn and assayed for the appearance of the markers. Lactate dehydrogenase and glutamate dehydrogenase were determined by monitoring the oxidation of NADH [10,11]. β -Hexosaminidase was measured according to Barrett [12] using *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as a substrate. All results were normalized to the total enzyme activity of supernatants of cells homogenized in 10 mM-Tris/HCl, pH 7.4, containing 0.1% Triton X-100, and centrifuged for 5 min at 10000 *g*. K^+ was measured by flame-emission spectroscopy in samples diluted with 0.1% CsCl (Suprapur, Merck). $^{36}\text{Cl}^-$ was injected by means of a micropump [13].

Presentation of results

Ouabain binding and $^{86}\text{Rb}^+$ flux data are given as the means $\pm$ S.D. of ten separate determinations in individual oocytes. Correlation coefficients (*r*) were derived from linear regression analysis.

RESULTS

Permeabilization with digitonin and SDS

The concentration-dependent release by digitonin of marker enzymes and microinjected $^{36}\text{Cl}^-$ is shown in Fig. 1(a). The plasma-membrane permeability increased sharply at $\geq 3 \mu\text{M}$ -digitonin. After 30 min at 10 μM -digitonin and 5 $^\circ\text{C}$, most of the $^{36}\text{Cl}^-$ and the lactate dehydrogenase (cytosol) but less than 1% of the β -hexosaminidase (lysosomes) were liberated. Glutamate dehydrogenase (mitochondria) was not released by up to 300 μM -digitonin.

On subsequent incubation in digitonin-free medium at 25 $^\circ\text{C}$, a continuous release of β -hexosaminidase was noted in oocytes permeabilized at $> 10 \mu\text{M}$ -digitonin (results not shown). In contrast, the enzyme did not appear in the medium when oocytes were treated by no more than 10 μM -digitonin, as demonstrated in Fig. 1(b). Since the release of the cytosolic markers was complete at this concentration (Fig. 1a), 10 μM -digitonin was considered to be optimal to permeabilize the plasma membrane with little change in the permeability of intracellular membranes. To disrupt inner membranes, digitonin-treated oocytes were exposed to the nonspecific detergent SDS. The time-course of the SDS-induced release of the lysosomal marker β -hexosaminidase is illustrated in Fig. 1(b).

Fig. 1. Permeabilization of oocyte compartments by detergents

(a) Oocytes were exposed to the indicated concentrations of digitonin for 30 min at 5 $^\circ\text{C}$. Both media and Triton X-100-treated cell homogenates were assayed for enzyme activities and $^{36}\text{Cl}^-$. (b) Oocytes permeabilized at 10 μM -digitonin for 30 min were reincubated at 25 $^\circ\text{C}$ in the Na/Tris medium containing 1 mM-ATP in the absence (○) and presence (●) of 0.02% SDS. β -HA, β -hexosaminidase; LDH, lactate dehydrogenase; GDH, glutamate dehydrogenase.

Fig. 2. Effect of SDS on ouabain binding to digitonin-permeabilized oocytes

Oocytes permeabilized with 10 μM -digitonin were reincubated at 25 $^\circ\text{C}$ in the absence (○) or presence (■, ●) of 0.02% SDS. (a) Time course of binding at 50 nM- $^{3\text{H}}$ ouabain. (b) Scatchard plots derived after 4 h of incubation at 25 $^\circ\text{C}$. $^{3\text{H}}$ ouabain ranged between 1 and 50 nM. Contaminating K^+ in the medium was 0.1 mM, as determined at the end of the incubation. Inset: Binding of $^{3\text{H}}$ ouabain (50 nM) to digitonin-permeabilized oocytes as a function of SDS concentration.

Ouabain binding to permeabilized oocytes

Since ouabain binding is K^+ -sensitive, oocytes were permeabilized and simultaneously depleted of K^+ by incubation in a K^+ -free medium. During the permeabilization procedure, internal K^+ declined from 51 to 10 nmol per oocyte. At 50 nM- $^{3\text{H}}$ ouabain and 1 mM-ATP, specific binding to oocytes treated in this way accounted for about 90% of total binding in the NaCl/Tris medium. Specific ouabain binding increased when digitonin-permeabilized oocytes were exposed to increasing concentrations of SDS (inset of Fig. 2b). In order to

determine equilibrium conditions, the time course of ouabain binding was examined. An apparent plateau of binding was reached after 4 h at 25 °C both with and without 0.02 % SDS, as shown in Fig. 2(a).

The Scatchard graphs shown in Fig. 2(b) indicate that an optimal concentration of 0.02 % SDS had little effect on the apparent dissociation constant (K_d), but increased the ouabain binding capacity (B_{max}) by a factor of two. According to the Scatchard graphs of three independent experiments, SDS induced an increase in B_{max} of $105 \pm 15\%$. The K_d values derived from the slopes of the straight lines ($r > 0.983$) averaged 3.7 ± 0.7 nM and 2.6 ± 0.3 nM ($\pm$ s.d.) in the absence and presence respectively of SDS. Both in the absence and presence of SDS, ouabain binding was increased by added ATP and reduced by K^+ , as shown in Table 1.

Surface versus subcellular binding of ouabain

To check whether permeabilization of the plasma membrane uncovers Na^+/K^+ pumps, B_{max} values of intact and permeabilized oocytes were compared. Since *Xenopus* oocytes internalize ouabain by endocytosis [7], cells were pulse-labelled with [3H]ouabain and then assayed for residual uptake of $^{86}Rb^+$. Results are presented in Table 2. The calculation is based on the assumption that the residual uptake of $^{86}Rb^+$ indicates the fraction of pumps not labelled by ouabain. The number of ouabain binding sites per cell surface estimated from these data agreed well with the B_{max} derived from the Scatchard analysis of oocytes from the same batch permeabilized at 10 μ M-digitonin.

In another series of experiments, the Na^+/K^+ pumps on

the surface of intact oocytes were masked with unlabelled ouabain. Once bound, ouabain dissociates slowly from the Na^+/K^+ pumps of oocytes ($t_{1/2} \sim 2$ days at 25 °C). The prelabelled oocytes were permeabilized and assayed for [3H]ouabain binding. Controls with intact oocytes were run in parallel. As illustrated in Fig. 3, [3H]ouabain binding to intact and permeabilized cells was reduced to the same extent. This indicates that the sites labelled in digitonin-permeabilized oocytes must face to the external medium, because they could be blocked from outside the intact cells. In contrast, the sites exposed by treatment with SDS (Fig. 3) or high concentrations of digitonin (results not shown) could not be blocked by pulse-labelling of the surface Na^+/K^+ pumps with cold ouabain.

Detergent-induced ouabain binding and release of β -hexosaminidase

Specific ouabain binding increased as a linear function of the digitonin concentration used for permeabilization, as illustrated in Fig. 4(a). In contrast, ouabain binding in the presence of 0.02 % SDS was independent of the

Table 1. Effect of ATP and K^+ on ouabain binding to permeabilized oocytes

Digitonin-treated cells were incubated for 4 h in the NaCl/Tris medium containing 50 nM-[3H]ouabain and additions as indicated. Nonspecific binding (14 ± 4 c.p.m. per cell) was subtracted from the data.

Additions	Ouabain binding (c.p.m./oocyte)	
	No SDS	0.02 % SDS
No K^+ , no ATP	29 ± 14	166 ± 18
50 mM- K^+ , no ATP	7 ± 3	18 ± 7
No K^+ , 1 mM-ATP	116 ± 13	287 ± 32
50 mM- K^+ , 1 mM-ATP	11 ± 6	58 ± 8

Table 2. Ouabain binding capacity of intact and permeabilized oocytes

Total and ouabain-insensitive $^{86}Rb^+$ uptake amounted to 13.8 ± 2.5 and 3.7 ± 1.5 fmol/min per oocyte respectively. B_{max} of intact cells was calculated as the amount of ouabain required to completely block active $^{86}Rb^+$ uptake. The B_{max} of oocytes permeabilized at 10 μ M-digitonin was derived from Scatchard plots according to the protocol described in the legend to Fig. 2(b).

Intact oocytes			Digitonin-treated oocytes	
Ouabain bound (fmol/oocyte)	Inhibition of Rb^+ uptake (%)	Calculated B_{max} (fmol/oocyte)	B_{max} (fmol/oocyte) (no SDS)	B_{max} (fmol/oocyte) (0.02 % SDS)
5.2 ± 0.6	78 ± 7	6.7 ± 0.7	6.2 ± 0.9	17.4 ± 1.2

Fig. 3. [3H]Ouabain binding to cells with ouabain-masked surface binding sites

Intact oocytes were preincubated with 10 μ M unlabelled ouabain for 10 min prior to permeabilization at 10 μ M-digitonin. Subsequent binding at 0.2 μ M-[3H]ouabain was terminated after 20 min at 25 °C. Intact cells were incubated in parallel.

Fig. 4. Effect of digitonin on ouabain binding and release of β -hexosaminidase

(a) Oocytes permeabilized at the indicated concentrations of digitonin were assayed for ouabain binding (4 h at 25 °C). (b) β -Hexosaminidase released is the sum of the enzyme activities (as % of total) present in both the permeabilization and the incubation media. Ouabain binding data are the same as in (a). β -HA, β -hexosaminidase.

digitonin concentration in the permeabilization medium (Fig. 4a). The latency of β -hexosaminidase (Fig. 1a) suggested that high concentrations of digitonin disrupted inner membranes similar to SDS (Fig. 1b). A linear relationship ($r = 0.986$) could be established between the amount of ouabain bound specifically and the sum of the total β -hexosaminidase released at the end of both the permeabilization procedure and the ouabain binding assay. By comparison, the total β -hexosaminidase released from the digitonin-permeabilized oocytes treated by SDS ranged between 26 % and 39 % (Fig. 4b).

DISCUSSION

The Na^+/K^+ -ATPase has been traditionally regarded as a marker of the plasma membrane. However, more recent studies suggested that a significant fraction of the total Na^+/K^+ -ATPase resides in the interior of some types of cells. For instance, up to 55 % of the newly synthesized Na^+/K^+ -ATPase of chick sensory neurons did not appear on the cell surface but remained in an internal pool [3]. In membranes of rat exorbital lacrimal gland, the Na^+/K^+ -ATPase was associated with two distinct membrane populations, one of which appeared to be derived from the Golgi complex [14]. The Golgi apparatus was also demonstrated in an immunocytochemical study to bind a monoclonal antibody directed against the Na^+/K^+ -ATPase [15].

The present data suggest the existence of a large intracellular pool of Na^+/K^+ pumps in *Xenopus* oocytes. Evidence is as follows: (i) B_{max} was higher by a factor of two in SDS-treated cells than in intact or digitonin-permeabilized oocytes (Table 2); (ii) masking of surface Na^+/K^+ pumps by ouabain failed to inhibit SDS-induced [^3H]ouabain binding (Fig. 3); (iii) ouabain binding increased in proportion to the release of the lysosomal marker β -hexosaminidase (Fig. 4b).

The results presented in Figs. 1(a) and 4(b) agree with the view that digitonin acts selectively on the plasma membrane only in a narrow range of detergent concen-

tration [16]. The close correspondence of B_{max} values in intact and digitonin-treated cells (Table 2) indicates that permeabilization by itself did not uncover ouabain binding unless inner membranes were disrupted (cf. Fig. 4). These findings argue against the possibility that detergents simply activate cryptic ouabain binding sites on the cell surface. Since SDS-induced binding of ouabain was supported by ATP (Table 1), the intracellular Na^+/K^+ pumps seem to be capable of hydrolysing ATP and to adopt the E_2P conformation. This suggests that the internal Na^+/K^+ pumps are in functional state.

Prophase-arrested oocytes internalize severalfold more ouabain molecules than are required for complete inhibition of active K^+ transport across the plasma membrane [7]. A similar phenomenon in HeLa cells has been attributed to a continuous translocation of Na^+/K^+ pumps from the cell surface into lysosomes [17]. Association of Na^+/K^+ pumps with lysosomes in oocytes might be deduced from the close relationship of ouabain binding and the release of the lysosomal marker β -hexosaminidase on permeabilization with digitonin (Fig. 4b). Maximal lysosomal binding of ouabain under conditions when only a fraction of the total β -hexosaminidase present in oocytes was released (Fig. 4) might be explained by lysosome heterogeneity. At least two distinct populations of lysosomes have been identified in oocytes by sucrose density-gradient fractionation [18]. It might be speculated that the internal Na^+/K^+ pumps are located exclusively in one type of lysosome, the permeabilization of which would uncover all internal Na^+/K^+ pumps, but liberate only a part of the total β -hexosaminidase present in the oocytes. Alternatively, the internal Na^+/K^+ pumps might reside in organelles that become leaky under the same conditions as the lysosomes. It has been proposed that Na^+/K^+ pumps internalized from the cell surface return to the Golgi apparatus for reutilization mixed with newly synthesized enzymes [4]. We speculate that a recycling of Na^+/K^+ pumps also takes place in oocytes. Localization in the Golgi apparatus would suggest that the intracellular Na^+/K^+ pumps are in principle available for transit to the cell surface. The store might serve for the rapid delivery of Na^+/K^+ pumps to the surface when the cell starts dividing after fertilization.

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