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Up-regulation of sodium pump activity in *Xenopus laevis* oocytes by expression of heterologous β1 subunits of the sodium pump

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Recent evidence suggests that the β subunit of the Na^+ pump is essential for the α subunit to express catalytic activity and for assembly of the holoenzyme in the plasma membrane. We report here that injection into *Xenopus laevis* oocytes of cRNAs specific for β1 subunit isoforms of the Na^+ pump of four species (*Torpedo californica*, chicken, mouse and rat) causes a time-dependent increase in the number of ouabain-binding sites, both in the plasma membrane and in internal membranes. Expression of the β1 subunit of the Na^+ pump of mouse and rat in the oocytes could be substantiated by immunoprecipitation using a polyclonal antiserum against the mouse β1 subunit. Scatchard analysis in permeabilized cells disclosed that the affinity for ouabain is unchanged after expression of each of the β1 subunits. A proportional increase in ouabain-sensitive $^{86}Rb^+$ uptake indicates that the additionally expressed ouabain-binding sites on the cell surface represent functional Na^+ pumps. The findings support the concept of Geering. Theulaz, Verrey, Häuptle & Rossier [(1989) Am. J. Physiol. **257**, C851–C858] that β1 subunits expressed in oocytes associate with an excess of endogenous α subunits of the Na^+ pump to form a hybrid enzyme. In addition, all of the β1 isoforms investigated in the present study were also capable of combining with the co-expressed α1 subunit of the *Torpedo* Na^+ pump to produce a functional enzyme. Injection of cRNA encoding for the *Torpedo* α1 subunit alone had no effect on the ouabain-binding capacity of the surface and intracellular membranes of the oocyte.

INTRODUCTION

The Na^+ pump consists of a catalytic α subunit of about 100 kDa and a smaller glycosylated β subunit. The α subunit traverses the membrane seven or eight times; the binding region for ouabain and related cardiac glycosides are exposed extracellularly, whereas the ATP-binding and -phosphorylation sites are located on a large cytoplasmic loop. While the transport functions of the Na^+ pump can be attributed to the α subunit, the role of the β subunit is less clear. Some evidence suggests that the β subunit is essential for the maturation and assembly in the plasma membrane of the α subunit ([1,2]; see [3,4] for reviews). The two subunits are co-translationally and independently inserted into the membranes of the rough endoplasmic reticulum [5–7]. Immediately after synthesis, the α subunit is highly sensitive to trypsin [5] and unable to bind ouabain [8]. Acquisition of the capacity to undergo cation-dependent changes in conformation is associated with an increase in trypsin resistance and occurs in most cells within 20 min of synthesis [9]. In the *Xenopus laevis* oocyte, however, α subunits synthesized in large excess over β subunits retain their trypsin sensitivity [2]. This suggests that the excess over α subunits are immature and incapable of transporting Na^+ and K^+ [2]. Trypsin resistance can be induced by injection of cRNA encoding the β1 subunit of the Na^+ pump from kidney cells of *Xenopus laevis*, and is associated with enhanced Na^+ pump activity [2]. These results demonstrate clearly that the α subunit requires the β subunit in order to change to a conformation which possesses catalytic activity. The functional importance of the β subunit has also been inferred from the finding that expression of functionally active Na^+ pumps of *Torpedo californica* in *Xenopus* oocytes requires co-injection of cRNAs for both subunits. Injection of α-subunit-specific cRNA alone resulted in the expression of an inactive enzyme [10].

The α subunit, and most likely also the β subunit, of the Na^+-ATPase are encoded by a multigene family. Three isoforms of the α subunit (α1, α2, and α3; see [11] for a review) and three isoforms of the β subunit, β1 (see below), β2 [12,13] and β3 [13a], have been identified at both the mRNA and the protein level. cDNA clones for β1 subunits have been characterized in various species, including sheep kidney [14], pig kidney [15], electric organ of *Torpedo californica* [16], HeLa cells [17], rat kidney and brain [18,19], chicken [20] and mouse brain [21], and kidney cells of *Xenopus laevis* [22].

To examine whether β subunits of species other than *Xenopus* can associate with endogenous α subunits, we injected *Xenopus* oocytes with cRNAs encoding one of four different β1 subunits of Na^+ pumps previously cloned from the electroplax of *Torpedo californica* [10] and neuronal tissue of chicken [20], mouse [21] and rat [18]. We report here that expression of each of these exogenous β1 subunits alone significantly increased the net number of functional Na^+ pumps. In addition, all of these β1 subunits can also complex with co-expressed α subunits of the Na^+ pump of *Torpedo californica*. Some of these results have been presented in abstract form [23].

MATERIALS AND METHODS

Chemicals

[^{3}H]Ouabain (1.1, 0.62 and 0.67 TBq/mmol), ^{86}RbCl and [^{14}C]sucrose (0.37 GBq/mmol) were purchased from Amersham–Buchler; L-[^{35}S]methionine (approx. 40 TBq/mmol) was from New England Nuclear. Restriction enzymes were from Biolabs, except for *Nhe*I (Boehringer Mannheim) and *Sma*I (Stratagene). If not otherwise indicated, all other chemicals were purchased from Merck at analytical grade.

Abbreviations used: K_D, apparent dissociation constant; $B_{max.}$, ouabain-binding capacity; ORI, oocyte Ringer's solution; PBS, phosphate-buffered saline.

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cRNA synthesis

The cloning and sequencing of the $\beta 1$ subunit of the Na^+ pump from mouse brain has been described recently [21]. Other plasmids containing cDNA clones specific for the $\alpha 1$ or $\beta 1$ subunits of Na^+ pumps were generously provided by the following scientists: pGEMβ36 for the $\beta 1$ subunit of the rat brain Na^+ pump [18] from Dr. E. J. Benz and Dr. P. Graham, Yale University, New Haven, CN, U.S.A.; pEMBL18+containing cDNA for the $\beta 1$ subunit from chicken brain [20] from Dr. D. Fambrough, Johns Hopkins University, Baltimore, MD, U.S.A.; pSPNKAα for the $\alpha 1$ and pSPNKAβ1 for the $\beta 1$ subunit from *Torpedo californica* electroplax [10,16] from Dr. M. Kawamura, University of Occupational and Environmental Health, Kitakyushu, Japan. The full-length cDNA insert in pEMBL18+encoding the chicken $\beta 1$ subunit [20] was subcloned into the *Eco*RI site of pSPT19 (Pharmacia) to obtain $C_{\beta 1}$-pSPT19. pGEMβ36 was hydrolysed with *Sma*I, $C_{\beta 1}$-pSPT19, pSPNKAα and pSPNKAβ1 with *Sal*I and $M_{\beta 1}$-pGEM2, encoding the $\beta 1$ subunit of mouse, with *Bam*HI. Capped subunit-specific cRNAs were synthesized *in vitro* with SP6 RNA polymerase (Pharmacia) in the presence of 0.5 mM capping dinucleotide $m^7G(5')ppp(5')G$ (Biolabs) and 0.05 mM-GTP as described [24]. For synthesis of capped antisense cRNA complementary to the $\beta 1$ subunit of the rat, pGEMβ36 was linearized with *Nhe*I and transcribed with T7 RNA polymerase (Stratagene). After digestion of templates with RQ1 DNAase (Promega), the reaction mixture was extracted once with phenol/chloroform (1:1, v/v) and twice with chloroform. cRNAs were then precipitated twice with 0.7 M-ammonium acetate and 2.5 vol. of ethanol. After washing the final pellets with 70% ethanol, the cRNAs were dissolved in sterile water, divided into portions and stored at −196 °C until used. The concentration was quantified using the adsorbance reading at 260 nm (1 unit = 40 μg/μl). The $\beta 1$ subunit-specific cRNAs were not polyadenylated.

Isolation of oocytes and maintenance after cRNA injection

Ovarian lobes of *Xenopus laevis* (Xenopus Ltd., Redhill, Surrey, U.K.) anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 2 g/l; Sandoz, Basel, Switzerland) were surgically removed and disrupted into small clumps. Oocytes were released by overnight incubation at 20–22 °C with collagenase (1.0–1.5 units/ml; no. 17449; Serva, Heidelberg, Germany) in modified oocyte Ringer's solution (1 mM-K^+-ORI:90 mM-NaCl, 1 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$ and 10 mM-Hepes, pH 7.4) supplemented with penicillin (20 mg/l) and streptomycin (25 mg/l). After dissolution of collagenase, the medium was filtered (0.2 μm pore diameter). Complete release of follicular cells required subsequent incubation for 10–30 min in Ca^{2+}-free 1 mM-K^+-ORI without collagenase, followed by several washes in sterile 1 mM-K^+-ORI. Defolliculated oocytes of oogenesis stages V or VI [25] were manually selected, microinjected with 50 nl of cRNA (0.03–1 μg/μl) and kept at 17 °C in sterile 1 mM-K^+-ORI containing gentamycin (50 mg/l). Occasionally the entire absence of follicular cells was confirmed by fluorescence microscopy as described by Horrell *et al.* [26]. Oocytes injected with 50 nl of water or non-injected oocytes of the same cell batch were cultivated in parallel and served as controls. Medium was changed daily during cultivation.

Measurements of ouabain binding and $^{86}Rb^+$ uptake

If not indicated otherwise, cultivated oocytes were loaded with Na^+ by preincubation for 1 h at 21–23 °C in a Na^+-loading medium [27] nominally free of Ca^{2+} and K^+ (110 mM-NaCl, 10 mM-Tris, pH 7.4, with HCl), followed by a 15 min pre-incubation in K^+-free ORI. This treatment was performed to accelerate ouabain binding as described in the Results section. Surface binding of ouabain was assessed by incubating the Na^+-loaded cells in K^+-free ORI supplemented with 0.6–1.0 μM-[^{3}H]ouabain and 37 kBq of [^{14}C]sucrose/ml as an extracellular marker. After 20 min at 21–23 °C, unbound radioactivity was removed by washing. Since intact oocytes are almost impermeable to sucrose, inclusion of this extracellular marker allowed us to unequivocally distinguish inward leakage of ouabain from ouabain bound to the outer cell surface. Ouabain-binding data in cells that exhibited increased uptake of [^{14}C]sucrose (> 15 c.p.m./cell at 37 kBq of [^{14}C]sucrose/ml) were rejected. The cells were dissolved individually in 0.1 ml of 5% SDS and radioactivity was counted in 2 ml of Quickszint 2000 (Zinsser, Frankfurt/Main, Germany).

For measuring $^{86}Rb^+$ uptake, cells preloaded with Na^+ as above were incubated in K^+-free ORI containing 1 mM-$^{86}Rb^+$ (370 kBq/ml). Total and ouabain-insensitive uptake of $^{86}Rb^+$ were determined in the absence and presence respectively of 1 mM-ouabain. After 12–18 min at 21–23 °C, the cells were briefly washed in ice-cold medium and prepared for liquid scintillation counting as above. Rb^+ uptake was linear for at least 20 min under control and ouabain-inhibited conditions. Active $^{86}Rb^+$ transport was calculated by subtracting ouabain-insensitive uptake, which accounted for less than 10% of total uptake in Na^+-loaded oocytes.

For determination of surface and intracellular ouabain binding, cells not loaded with Na^+ were permeabilized with 10 μM-digitonin in 110 mM-NaCl/1 mM-$MgCl_2$/10 mM-EGTA/10 mM-Tris/HCl (pH 7.4) and assayed for ouabain binding at 25 °C in the absence and presence of 0.02% SDS exactly as described [28].

Metabolic labelling and immunoprecipitation of $\beta 1$ subunits of the Na^+ pump

For production of polyclonal antibodies against mouse $\beta 1$ subunit, a *Stu*I–*Ssp*I subfragment coding for amino acids 100–304 of the mouse $\beta 1$ subunit [21] was cloned into the blunt-ended *Bam*HI site of the bacterial expression vector pET3b [29] and sequenced. Proteins from 2 litres of induced culture were size-fractionated by preparative SDS/PAGE [30]. The induced $\beta 1$ subunit was excised from the gel, electroeluted, dialysed against phosphate-buffered saline (PBS) and used for immunization of rabbits. The IgG fraction was isolated by Protein A–Sepharose CL 4B chromatography (Pharmacia) and dialysed against PBS.

Oocytes injected with cRNA and non-injected controls were labelled with L-[^{35}S]methionine at 18 MBq/ml (0.37 MBq/oocyte) in ORI. In some experiments, tunicamycin (Sigma), an inhibitor of *N*-glycosylation (see [31] for a review) was co-injected at a concentration of 40 μg/μl with the cRNA and also included at 2 μg/μl in the labelling medium [32]. After 2 days at 17 °C, oocytes were extracted with Triton X-100 as described [33]. Aliquots of the extracts were diluted 5-fold with 60 mM-Tris/HCl, (pH 7.4)/190 mM-NaCl/6 mM-EDTA/1% Triton X-100, denatured with 3.3% SDS and incubated overnight at 4 °C with 80 μg of IgG/ml from the antiserum against the mouse $\beta 1$ subunit. Immune complexes were collected on Protein A–Sepharose CL 4B (Sigma) and electrophoresed in parallel with prestained protein molecular mass markers (Rainbow; Amersham) on 10% polyacrylamide gels in the presence of SDS [30]. For fluorography gels were fixed, soaked in Amplify (Amersham), dried and exposed to X-OMAT-AR film (Eastman Kodak Co.) at −80 °C.

Presentation of results

If not indicated otherwise, data are given as the means ± S.E.M. of 8–20 separate determinations in single oocytes. Ouabain-

binding data depicted in Figs. 2–4 correspond to approx. 90% of $B_{max.}$.

RESULTS

Apparent down-regulation of surface Na^+ pumps during oocyte cultivation

Reliable determination of the number of surface Na^+ pumps from ouabain binding to intact oocytes is complicated by the finding that the time course of ouabain binding exhibits two components: (i) a rapid component which is associated with inhibition of transmembrane Na^+/K^+ transport, and (ii) a slow component [34] which appears to be due to ouabain uptake via internalization of Na^+ pumps [28]. Since the time course of ouabain binding to the Na^+ pump is concentration-dependent, the influence of the second component on the estimate of the number of surface Na^+ pumps can be decreased by using a high concentration of [^{3}H]ouabain, allowing rapid labelling of most of the surface pumps. As is apparent from Table 1, 10 min of exposure to 0.6 μM-[^{3}H]ouabain was sufficient to inhibit close to 90% of ouabain-sensitive $^{86}Rb^+$ uptake, indicating that 90% of the surface pool of Na^+ pumps becomes labelled under these conditions. When incubation with [^{3}H]ouabain was continued, many more ouabain-binding sites were disclosed (Table 1). Since these additional sites cannot be attributed to inhibition of Rb^+ uptake, it is concluded that prolonged incubation with [^{3}H] ouabain will lead to an overestimation of the surface density of Na^+ pumps.

Table 1. Time course of ouabain binding and inhibition of $^{86}Rb^+$ uptake

Freshly isolated oocytes were incubated with and without 0.6 μM-[^{3}H]ouabain in K^+-free ORI. At the indicated times, the cells were washed and assayed for $^{86}Rb^+$ uptake at 1 mM-RbCl with and without 1 mM-ouabain. In the control cells not exposed to [^{3}H]ouabain, total and ouabain-insensitive $^{86}Rb^+$ uptake averaged 21 ± 1 and 0.4 ± 0.1 fmol/min per oocyte respectively, as assayed at each of the indicated times. $B_{max.}$ was calculated as the amount of ouabain required to completely block active $^{86}Rb^+$ uptake. The calculation is based on the assumption that ouabain binds in a 1:1 stoichiometry to a single class of sites and that the inhibition of $^{86}Rb^+$ uptake is directly proportional to the number of Na^+ pumps labelled with ouabain.

Time	Ouabain bound (fmol/oocyte)	Inhibition of Rb^+ uptake (%)	Calculated $B_{max.}$ (fmol/oocyte)
10 min	22.4 ± 0.8	87 ± 2	25.9 ± 1.0
50 min	33.6 ± 1.0	97 ± 1	34.6 ± 1.0
5 h	38.4 ± 1.2	99 ± 1	38.8 ± 1.2

In previous experiments we used oocytes as soon as possible after completing collagenase treatment. Cultivation of defolliculated oocytes for several days as required in the present study caused a considerable decrease in the apparent number of sites disclosed by pulse-labelling, as illustrated in Fig. 1(*a*). Within 1 day of cultivation ouabain binding had decreased to about 20% of its initial value. Lowered sensitivity towards ouabain rather than an actual decrease in the number of surface Na^+ pumps is suggested by the finding that 0.6 μM-[^{3}H]ouabain blocked 90% of active $^{86}Rb^+$ uptake in freshly isolated cells (cf. Table 1), but only 20–40% in cultivated oocytes (results not shown). In addition, active $^{86}Rb^+$ uptake itself was decreased in the cultivated cells, whereas ouabain-insensitive $^{86}Rb^+$ uptake determined at 1 mM unlabelled ouabain was not significantly affected. The down-regulation of ouabain binding could be prevented by keeping the oocytes in K^+-free ORI (Fig. 1*a*). Since internal Na^+ increases when the Na^+ pump is blocked in the absence of external K^+, it was tempting to assume that high sensitivity for ouabain depended on a high internal Na^+ concentration. The internal Na^+ concentration determined with Na^+-selective electrodes averaged 5.8 ± 0.2 mM (9 cells) and 20.6 ± 2.0 mM (5 cells) in oocytes cultivated for 2 days in the presence and absence respectively of 1 mM-K^+. Consistent with a role for internal Na^+ in ouabain binding, microinjection of 10 mM-NaCl (final concentration, calculated by assuming an oocyte water space of 0.5 μl) into cultivated oocytes instan-

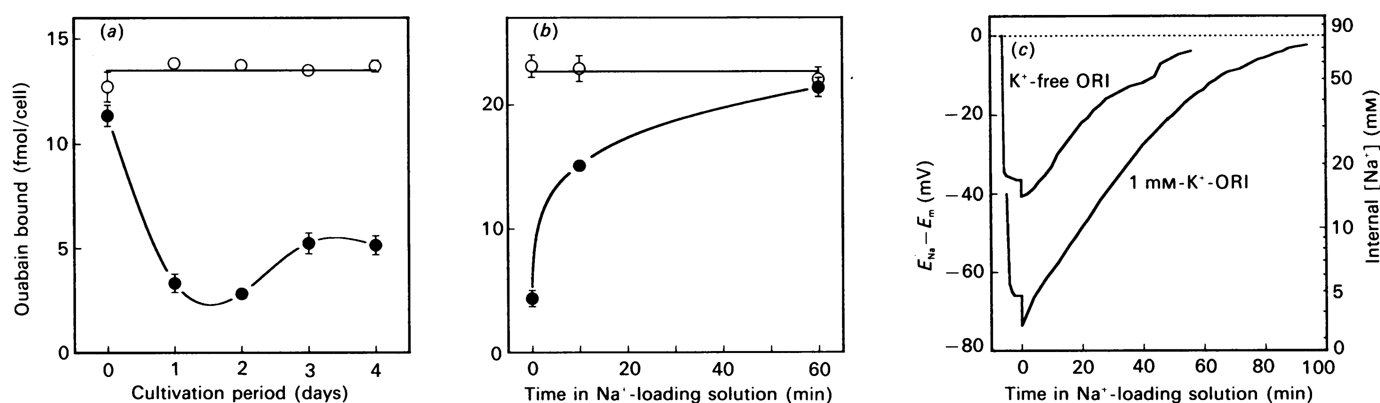

Fig. 1. Effect of Na^+ loading on ouabain binding to cultivated oocytes

(*a*) Defolliculated oocytes cultivated in 1 mM-K^+-ORI for the indicated numbers of days were assayed for ouabain binding without (●) or with (○) preincubation in the Na^+ loading medium. (*b*) Oocytes of the same cell batch were maintained either in K^+-free ORI (○) or in ORI supplemented with 1 mM-K^+ (●). After 3 days of cultivation at 17 °C, oocytes were preincubated in the Na^+-loading medium for the indicated times and were then labelled with [^{3}H]ouabain (1 μM) in K^+-free ORI for 20 min at 21 °C. (*c*) Internal Na^+ concentrations of oocytes exposed to $K^+/Ca^{2+}/Mg^{2+}$-free solution. Microelectrodes filled with a liquid neutral carrier (sodium ionophore I, no. 71176; Fluka) were calibrated in Ca^{2+}/Mg^{2+}-free solutions containing 10–100 mM-NaCl plus sufficient KCl to maintain a constant [Cl^-] of 110 mM. NaCl concentrations in the calibrating solutions were converted into activities assuming a Na^+ activity coefficient of 0.76. Electrodes polarized between 56 and 58 mV per unit increase in log $[Na^+]_o$. The Na^+ electrode was adjusted to zero in the Na^+-loading medium; superfusion of the chamber with Ca^{2+}-containing ORI generated an electrode potential of -5 to -10 mV. Oocytes cultivated for 3 days in K^+-free ORI or in 1 mM-K^+-ORI were impaled with a voltage electrode and a Na^+-sensitive electrode. At time zero, the bathing solution was switched from K^+-free ORI or 1 mM-K^+-ORI to the Na^+-loading medium.

Table 2. Effect of Na^+ loading on ouabain binding to intact and permeabilized oocytes

Oocytes cultivated in ORI for 2 days at 17 °C were preloaded with Na^+ as described in the Materials and methods section. Control cells were continuously maintained in ORI. Both control and Na^+-loaded cells were then washed with K^+-free ORI and immediately assayed for ouabain binding by a 20 min incubation at 0.6 μM-[^{3}H]ouabain in K^+-free ORI. Ouabain-binding rates in the intact cells preloaded with Na^+ are close to $B_{max.}$ (cf. Table 1). Subsets of cells from both preparations were additionally permeabilized with digitonin to determine surface and total binding of ouabain at 150 nM-[^{3}H]ouabain in the absence and the presence of 0.02 % SDS respectively. With K_D values of 20–25 nM (cf. Fig. 5), ouabain binding of the permeabilized cells corresponds to 86–88 % of $B_{max.}$.

Cells	Ouabain bound (fmol/cell)	
	Non-loaded	Na^+-preloaded
Intact cells	2.6±0.3	12.7±0.4
Permeabilized cells		
Surface	10.7±0.6	11.5±0.3
Total	22.9±0.4	22.0±0.6

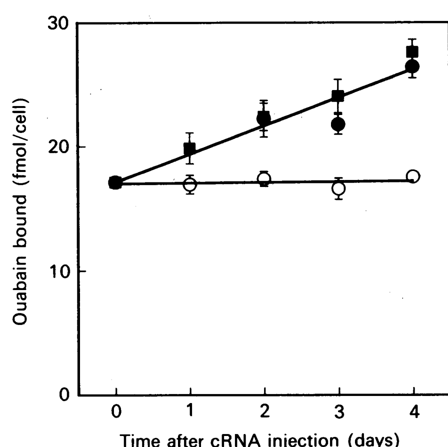

Fig. 2. Time course of the increase in the number of surface Na^+ pumps after injection of β1-subunit-specific cRNA

Oocytes injected with approx. 50 ng of cRNA encoding the β1 subunit of the Na^+ pump from chicken brain (●; cDNA insert 2.2 kb) or rat brain (■, cDNA insert 1.1 kb) were cultivated at 17 °C for the times indicated. Just before the ouabain-binding assay the cells were loaded with Na^+. Data were normalized to those of non-injected oocytes of the same cell batch (○), which were kept and assayed under identical conditions at the various times.

taneously raised the apparent number of ouabain-binding sites available for pulse-labelling from 3.3±0.4 to 13.0±0.7 fmol/cell. Ouabain binding in oocytes from the same batch of cells cultivated in K^+-free medium amounted to 16.6±0.5 fmol/cell, and was not significantly affected by injection of NaCl.

Fig. 1(*b*) illustrates that a large increase in ouabain binding could also be induced by exposing oocytes cultivated in the presence of 1 mM-K^+ to a K^+-free medium without added Ca^{2+} and Mg^{2+}. Ouabain binding to oocytes cultivated in K^+-free ORI was not affected by this treatment (Fig. 1*b*). The tracings of internal Na^+ concentration presented in Fig. 1(*c*) demonstrate that incubation of oocytes in the nominally Ca^{2+}- and Mg^{2+}-free medium elicits a fast and large increase in internal Na^+. Similar conditions have been shown in a variety of tissues, including mouse oocytes [35], to increase Na^+ permeability, presumably because Ca^{2+} channels become permeable for Na^+ when external Ca^{2+} is removed [36,37]. Comparison of the data of Figs. 1(*b*) and 1(*c*) suggests that about 20 mM internal Na^+ is required to ensure maximal ouabain binding in oocytes. Elevation of internal Na^+ has also been demonstrated in cultured chicken cardiac myocytes to be associated with a large increase in the apparent affinity of the Na^+ pump for ouabain [38]. Na^+ loading of oocytes also stimulated both the electrical current generated by the Na^+ pump [27] and ouabain-sensitive $^{86}Rb^+$ uptake.

To examine the possibility that Na^+ loading raises the number of surface Na^+ pumps by inducing exocytosis of internal Na^+ pumps, we permeabilized the plasma membranes of non-loaded and Na^+-loaded oocytes with digitonin. Permeabilization by 10 μM-digitonin allows the determination of the number of surface Na^+ pumps at a defined composition of the internal milieu without exposing intracellular binding sites for ouabain [28]. In addition, permeabilized cells do not internalize bound ouabain [28]. The results in Table 2 show that Na^+ loading before permeabilization had no effect on the number of ouabain-binding sites in the plasma membrane. The slightly lower number of surface sites derived from permeabilized cells compared with Na^+-loaded intact cells might be due to an overestimation of the number of surface Na^+ pumps in intact cells because of internalization of bound ouabain (cf. Table 1). By contrast, the number of sites determined in intact cells not loaded with Na^+ was significantly lower than in both the Na^+-loaded and the digitonin-permeabilized cells. In all the experiments described below, the cells were loaded with Na^+ just before the ouabain-binding assay was performed. Under these conditions, the number of ouabain-binding sites determined in non-injected oocytes turned out to be constant during several days of cultivation (Fig. 1*a*).

Expression of various exogenous β1 subunits increases surface binding of ouabain

When injected with cRNA encoding the Na^+ pump β1 subunit of chicken brain or rat brain, *Xenopus* oocytes showed increased numbers of Na^+ pumps in the plasma membrane (Fig. 2). The increase was already significant 1 day after injection. In the experiment described in the legend to Fig. 2, ouabain-binding sites continued to increase in number for up to at least 4 days after injection, but in other experiments a plateau was attained after 3 days. In all further experiments the oocytes were examined 2–3 days after cRNA injection. Since injection of either antisense cRNA complementary to one of the β1 subunits (Table 3) or water did not influence ouabain binding in three different experiments, non-injected oocytes were usually used as controls.

Fig. 3 shows the dose-dependence of the effect of β1-subunit-specific cRNAs on surface binding of ouabain. As little as 4–6 fmol of cRNA was sufficient to induce 4–7 fmol of additional ouabain-binding sites. The dose–response curves could be fitted by a rectangular hyperbola, with half-maximal effects of cRNA on ouabain binding at 4 fmol/cell (rat β1 subunit) and 6 fmol/cell (chicken β1 subunit). The level of newly expressed surface binding sites approached a maximal value when about 50 fmol (20–40 ng) of cRNA was injected per oocyte. With this amount of cRNA injected into oocytes from the same batch, the effects of all four β1-subunit-specific cRNAs were almost identical (results not shown). By contrast, with oocytes from different females, the relative increases in ouabain binding varied between 30 and 80 %.

Na^+ pumps induced by β1 subunit expression are functional

To examine whether the Na^+ pumps established by the expression of exogenous β1 subunits are functional, we measured the ouabain-sensitive uptake of $^{86}Rb^+$ and the pump-mediated current in addition to surface binding of ouabain. Fig. 4(*a*)

Table 3. Injection of α-subunit cRNA alone or of antisense β1 subunit cRNA has no effect on ouabain binding

Oocytes were injected or not with the indicated cRNAs and cultivated for 2 days at 17 °C before being permeabilized at 10 μM-digitonin. Surface and total binding of ouabain was determined in the absence and presence respectively of 0.02 % SDS. Data were normalized to those of non-injected control oocytes of the same cell batch cultivated and assayed under identical conditions. Ouabain binding (fmol/cell) to non-injected oocytes of the two cell batches used amounted to 7.1 ± 0.2 and 15.8 ± 0.4 (rows 1–3) and to 7.7 ± 0.2 and 18.0 ± 0.4 (rows 4 and 5) in the absence and presence respectively of SDS. Statistical significance was evaluated by the unpaired (two-tailed) Student's *t* test. *Significantly different from controls, $P < 0.0001$. $T_{\beta 1}$ and $R_{\beta 1}$, β1 subunits of the Na^+ pumps of *Torpedo* and rat respectively; $T_{\alpha 1}$, α1 subunit of the sodium pump of *Torpedo*.

cRNA injected per cell	Surface binding	Total binding
$T_{\alpha 1}$ (50 ng)	1.07 ± 0.02	1.05 ± 0.05
$T_{\beta 1}$ (30 ng)	1.70 ± 0.05*	1.61 ± 0.04*
$T_{\alpha 1}$ (25 ng) + $T_{\beta 1}$ (15 ng)	6.93 ± 0.45*	3.55 ± 0.27*
$R_{\beta 1}$ sense (30 ng)	1.36 ± 0.03*	1.40 ± 0.05*
$R_{\beta 1}$ antisense (30 ng)	0.96 ± 0.02	0.96 ± 0.03

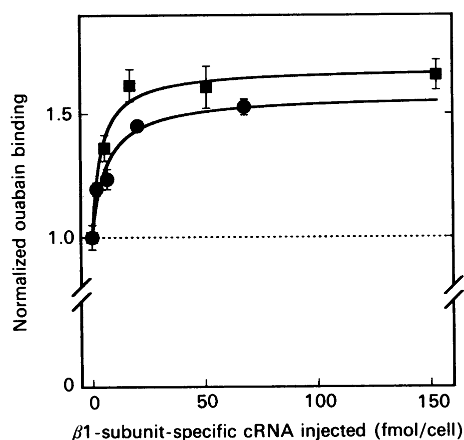

Fig. 3. Concentration-dependence of the effect of β1-subunit-specific cRNAs on surface binding of ouabain

At 2 days after injection of β1-subunit-specific cRNAs the cells were loaded with Na^+ and assayed for ouabain binding. Data were normalized to those of non-injected oocytes of the same cell batch (= 1.0), which were handled in parallel. Lines drawn through data points are non-linear least-squares fits to a rectangular hyperbola. ■, Expression of β1 subunit of rat brain Na^+ pump: half-maximal effect at 4.1 ± 1.3 fmol of cRNA; maximal increase of 68 ± 4 % corresponding to 8.4 ± 0.5 fmol of ouabain-binding sites; ●, expression of β1 subunit of chicken brain Na^+ pump: half-maximal effect at 6.6 ± 2.5 fmol of cRNA; maximal increase of 58 ± 6 % corresponding to 14.6 ± 1.5 fmol of ouabain-binding sites.

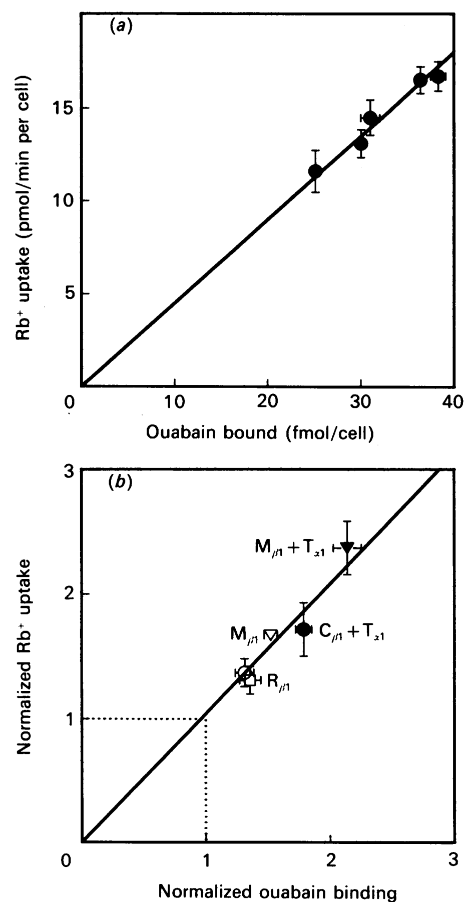

Fig. 4. Correlation between increased numbers of surface Na^+ pumps and active Rb^+ uptake after expression of exogenous β1 subunits

At 2 days after injection of cRNA, oocytes were loaded with Na^+ and assayed either for $^{86}Rb^+$ uptake with and without 1 mM-ouabain or for ouabain binding. (*a*) Oocytes were injected with 1.5–50 ng of cRNA for the β1 subunit of the chicken Na^+ pump. (*b*) Oocytes were injected either with 50 ng of cRNA for the indicated β1 subunit alone (open symbols) or co-injected with 15–25 ng of cRNA for the β1 subunit plus 15–25 ng for the *Torpedo* α1 subunit (filled symbols). Data were normalized to those of non-injected control oocytes of the same cell batch (= 1.0) cultivated and assayed under identical conditions. $C_{\beta 1}$, $M_{\beta 1}$, $R_{\beta 1}$ and $T_{\beta 1}$ are β1 subunits of the Na^+ pumps of chicken, mouse, rat and *Torpedo* respectively; $T_{\alpha 1}$, α1 subunit of the Na^+ pump of *Torpedo*.

illustrates that the number of surface ouabain-binding sites and ouabain-sensitive Rb^+ uptake were closely correlated after injection of oocytes with various amounts of chicken β1 subunit cRNA. Moreover, when maximal effective doses of the various β1-subunit-specific cRNAs were injected, ouabain-sensitive Rb^+ uptake increased in proportion to the number of surface ouabain-binding sites (Fig. 4*b*). Increased Na^+ pump activity caused by expression of the β1 subunit of rat brain was also indicated by electrical measurements under voltage-clamp conditions, showing that a 1.8-fold increase in the $B_{max.}$ for ouabain led to a 2-fold increase in pump-mediated current (results not shown). A further large increase in Na^+ pump activity could be induced by co-injection of any one of the β1-subunit-specific cRNAs with a cRNA encoding the *Torpedo californica* α1 subunit (Fig. 4*b*). Even larger increases in the ouabain-binding capacity and Na^+ pump activity could be elicited by increasing the amount of injected α subunit cRNA. For instance, co-injection of 50 ng of α subunit cRNA together with 25 ng of *Torpedo* or rat β1 subunit cRNA into cells from the same female raised the number of ouabain-binding sites from 4.7 to 111 ± 8 fmol/cell and from 4.6 to 108 ± 10 fmol/cell respectively. The electrical current generated by the Na^+ pump increased 3.7-fold and active Rb^+ uptake by 4.1-fold in the oocytes injected with *Torpedo* α subunit cRNA together with rat β1 subunit cRNA (results not shown). The findings demonstrate that expression of β1 subunits alone is sufficient to increase Na^+ pump activity, and that the various β1 subunits are all approximately equipotent in complexing with the *Torpedo* α subunit to form additional functional Na^+ pumps.

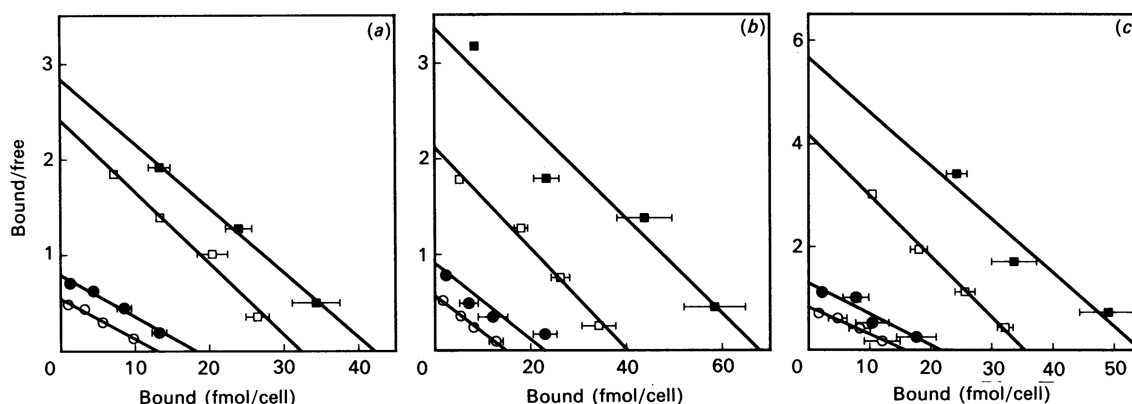

Fig. 5. Scatchard plots of ouabain binding to permeabilized oocytes expressing exogenous β1 subunits of the Na+ pump

Oocytes were injected with approx. 30 ng of cRNA specific for the β1 subunit of the Na+ pump (●, ■) of *Torpedo californica* electroplax (*a*), chicken brain (*b*) or mouse brain (*c*). After 2–3 days of cultivation the cells were permeabilized with digitonin and assayed for ouabain binding in the absence (○, ●) or presence (□, ■) of 0.02 % SDS. The free [³H]ouabain concentration ranged between 1 and 50 nM at the end of the incubation. Results are means ± S.D. of between 8 and 12 separate determinations in individual oocytes of typical experiments. Non-injected oocytes (○, □) were assayed in parallel.

Up-regulation of surface and intracellular ouabain-binding capacity by β1 subunits

Since *Xenopus* oocytes have a large pool of intracellular Na+ pumps [28], we investigated whether the increase in the number of surface ouabain-binding sites resulted from the recruitment of internal ouabain-binding sites or from a net increase in the total number of sites. For this purpose, oocytes were permeabilized with detergents 2–3 days after injection with cRNA specific for the various exogenous β1 subunits. In 13 independent experiments, expression of the β subunit of the *Torpedo* Na+ pump increased the number of ouabain-binding sites by 40 ± 3 % on the cell surface and by 43 ± 4 % in the cell interior, as assessed by permeabilization with digitonin alone or with digitonin plus 0.02 % SDS respectively. Sole injection of the cRNA for the α subunit of the *Torpedo* Na+ pump affected neither surface nor total binding of ouabain (Table 3). Scatchard analysis indicated that the effects of the *Torpedo* β subunit on ouabain binding were caused by an augmentation in the total binding capacity ($B_{max.}$), as illustrated in Fig. 5(*a*). As is apparent from the unchanged slopes of the Scatchard plots, the exogenous β subunit did not affect the ouabain sensitivity of the Na+ pump. Similar Scatchard plots were obtained after expression of the Na+ pump β1 subunit from chicken brain (Fig. 5*b*) and mouse brain (Fig. 5*c*).

Detection by immunoprecipitation of expressed β1 subunits from mouse and rat

A polyclonal antiserum generated against a polypeptide comprising amino acids 100–304 of the mouse β1 subunit recognized a 45 kDa protein in denatured extracts of [35S]methionine-labelled oocytes injected with cRNA for the mouse β1 subunit (Fig. 6, lane 2). A protein of the same apparent molecular mass could be immunoprecipitated from [35S]methionine-labelled oocytes injected with cRNA for the rat β1 subunit (Fig. 6, lane 5), which has 97 % sequence similarity to the mouse β1 subunit. The antibody did not cross-react with the β1 subunit of chicken expressed in oocytes (Fig. 6, lane 4).

In the presence of tunicamycin, an inhibitor of *N*-glycosylation [31], most of the mouse β1 subunit migrated at a lower apparent molecular mass of 35 kDa (Fig. 6, lane 3), close to the 35.2 kDa calculated for the core polypeptide from the amino acid sequence deduced from the cDNA [21]. In oocytes of the same batch, injection of the same cRNA increased the total number of ouabain-binding sites by 48 ± 9 and 46 ± 6 % in the absence and

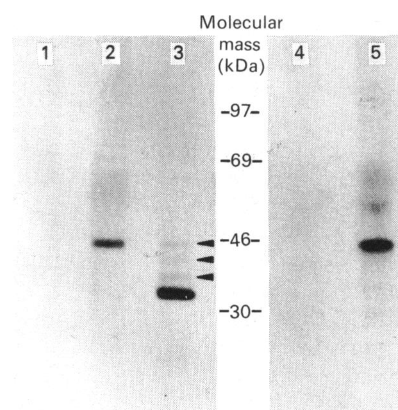

Fig. 6. Immunoprecipitation of Na+ pump β1 subunits of mouse and rat expressed in *Xenopus* oocytes

Oocytes were microinjected with cRNA and cultivated for 2 days in the presence of [35S]methionine. Triton X-100 extracts were prepared, denatured with SDS and immunoprecipitated with antibody against a polypeptide comprising amino acids 100–304 of the mouse β1 subunit. Immunoprecipitates from 1.7 homogenized oocytes per track were analysed by SDS/PAGE and fluorography. Samples are non-injected oocytes (lane 1), and oocytes injected with cRNA for the β1 subunit of mouse (lanes 2 and 3), chicken (lane 4), or rat (lane 5) Na+ pump. Some of the oocytes were labelled in the presence of tunicamycin to inhibit *N*-glycosylation (lane 3).

presence respectively of tunicamycin. Apparently, *N*-glycosylation of the expressed β1 subunit is not required for the rise in the number of Na+ pumps. The three additional bands marked by the arrows in Fig. 6, lane 3, correspond presumably to distinct glycosylation states of the β1 subunit. This suggests that the three potential glycosylation sites predicted from the cDNA [21] are all used.

DISCUSSION

How might expression of β1 subunits increase Na+ pump activity?

Recent evidence suggests that the Na+ pump of *Xenopus* oocytes is regulated by endocytosis [39] and exocytosis [40]. In principle, up-regulation of Na+ pump activity after microinjection of cRNAs encoding β1 subunits (Figs. 2–4) could result from

insertion of Na^+ pumps pre-existing in internal membranes into the plasma membrane. However, the net increases in the numbers of both internal and surface binding sites for ouabain revealed by Scatchard analysis (Fig. 5) clearly excludes this possibility. Since the ouabain-binding sites and cation-binding sites reside on the α subunit [41–45], expression of β1 subunits by itself cannot generate functional Na^+ pumps. A likely explanation for the effect of the exogenous β subunits comes from recent findings of Geering *et al.* [2] showing that *Xenopus* oocytes contain endogenous α subunits in excess of β subunits. The overexpressed α subunits are trypsin-sensitive, but acquire trypsin resistance after injection of cRNA encoding for the Na^+ pump β1 subunit from kidney cells of *Xenopus* [2], and Na^+ pump activity increases in parallel. Apparently, the additionally expressed β subunits associate with endogenous α subunits and induce a structural reorganization such that the Na^+ pumps which are formed become functional. Present findings demonstrate that the capability of the *Xenopus* β1 subunit is shared by β1 subunits from four species, suggesting that all of them are capable of forming interspecies hybrid Na^+ pumps with endogenous α subunits of the oocyte. The non-selectivity of the hybrid formation between the various β1 subunits and the α subunit is supported by the finding that functional Na^+ pumps could also be induced by co-injection of cRNAs specific for the *Torpedo* α subunits together with one of the β1 subunits from the other species (Fig. 4*b*). In other cells, expression of active Na^+ pumps from cDNA specific for either the α or the β subunit has also been attributed to the formation of hybrid Na^+ pumps from exogenous α subunits and endogenous β subunits [45,46] and *vice versa* [20].

It should be emphasized that the pool of immature α subunits in *Xenopus* oocytes described by Geering *et al.* [2] is unlikely to correspond to the pool of internal Na^+ pumps disclosed by permeabilization of the oocytes [28]. There are several reasons for this conclusion. (i) Immature α subunits fail to undergo cation-dependent conformational changes [2], whereas ouabain binding to internal Na^+ pumps is affected by ATP and K^+, as expected for a functional enzyme [28]. (ii) Expression of exogenous β subunits increases the internal pool of functional Na^+ pumps (Fig. 5), most likely at the expense of non-functional α subunits. (iii) cRNA coding the α subunit of the Na^+ pump of *Torpedo californica* has been shown to be translated in the *Xenopus* oocyte even without co-injection of cRNA for the β subunit [10], but failed to elevate intracellular ouabain binding (Table 3).

We are thus confronted with the complex situation that *Xenopus* oocytes possess at least three pools of Na^+ pumps, as outlined in Fig. 7. The sizes of the pools are determined by the availability of β subunits. Any increase in the expression of β subunits will diminish the number of non-functional α subunits and increase the number of functional Na^+ pumps, and vice versa. In the scheme in Fig. 7, it is assumed that surface Na^+ pumps are delivered to the plasma membrane via the intracellular pool of Na^+ pumps disclosed in the permeabilized cells. It is equally possible, however, that an additional compartment is involved in the assembly of Na^+ pumps in the plasma membrane and that the internal pool is formed subsequently by endocytosis of surface Na^+ pumps. Rapid cycling of internal and surface Na^+ pumps is inferred from internalization of bound ouabain (Table 1) and might be correlated with the high endocytotic activity of the *Xenopus* oocyte.

Apparent lack of species differences in the effects of the various β1 subunits

All four β1-subunit-specific cRNAs investigated in the present study elicited almost the same net increase in the number of Na^+ pumps provided that oocytes from the same female were used for

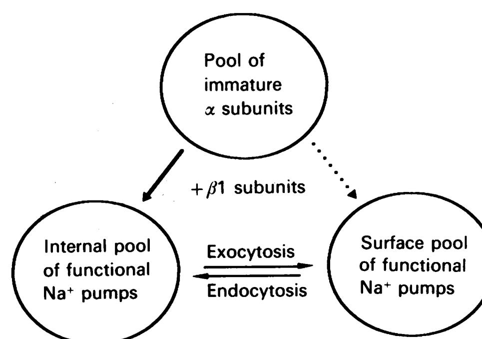

Fig. 7. Model for up-regulation of Na^+ pump activity by β1 subunit expression

Immature α subunits present in excess over β subunits in the *Xenopus* oocyte [2] complex with β_1 subunits expressed by cRNA injection and form interspecies hybrid Na^+ pumps which are functional. See the text for further explanation.

expression. Moreover, the measurements of Rb^+ uptake (Fig. 4*b*) and pump-generated current (results not shown) suggested that all of the hybrid Na^+ pumps exhibit virtually the same turnover in the Na^+-loaded cells as the wild-type Na^+ pump. Comparison of our data with those of Geering *et al.* [2] indicates that the heterologous β1 subunits are as effective in raising the number of ouabain-binding sites as the homologous β1 subunits of *Xenopus laevis* itself. The derived amino acid sequence of the *Xenopus* β1 subunit [22] has 61–67% identity with the β1 subunit sequences of *Torpedo* [16], chicken [20], mouse, [21] and rat [18], and more than 32% of the amino acid substitutions between *Xenopus*, *Torpedo* and the mammalian species are conservative. This might account for the apparent non-selectivity of the β1 subunits in forming functional Na^+ pumps by combining either with α subunits of the host cell or with co-expressed α subunits of *Torpedo*.

Sole expression of β subunits as a putative mechanism for regulation of Na^+ pump activity

Precise control of the number of Na^+ pumps at the pre-translational level by co-ordinate synthesis of α and β subunits would require the simultaneous regulation of the two separate and unlinked genes coding for the two subunits (see [11] for review). The findings of Geering *et al.* [2] and the present study suggest that a rise in the number of Na^+ pumps can also be achieved by the easier regulation of the gene for the β subunit alone, provided that the cell possesses a pool of α subunits lacking β subunits. Such an up-regulation of Na^+ pump activity directed by the sole synthesis of β subunits has been recently reported to occur in LLC-PK_1 cells maintained at a low external K^+ concentration [47].

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