Electrophysiological Characterization of Ionic Transport by the Retinal Exchanger Expressed in Human Embryonic Kidney Cells

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ABSTRACT The retinal Na^+:Ca^{2+},K^+ exchanger cDNA was transiently expressed in human embrionic kidney (HEK 293) cells by transfection with plasmid DNA. The correct targeting of the expressed protein to the plasma membrane was confirmed by immunocytochemistry. The reverse exchange current (Ca^{2+} imported per Na^+ extruded) was measured in whole-cell voltage-clamp experiments after intracellular perfusion with Na^+ (Na_i^+, 128 mM) and extracellular perfusion with Ca^{2+} (Ca_o^{2+}, 1 mM) and K_o^+ (20 mM). As expected, the exchange current was suppressed by removing Ca_o^{2+}. Surprisingly, however, it was also abolished by increasing Na_o^+ to almost abolish the Na^+ gradient, and it was almost unaffected by the removal of K_o^+. Apparently, then, at variance with the exchanger in the rod outer segment, the retinal exchanger expressed in 293 cells acts essentially as a Na^+:Ca^{2+} exchanger and does not require K^+ for its electrogenic activity.

INTRODUCTION

The vertebrate photoreceptor Na^+:Ca^{2+},K^+ exchanger (Yau and Nakatani, 1984; Hodgkin et al., 1987) is important to phototransduction, because sustained exchange activity in the presence of light induces a fall in $[Ca^{2+}]_i$, which in turn triggers the cascade of events involved in the dark-state recovery and light adaptation of the photoreceptor (reviewed by McNaughton, 1995; Detwiler and Gray-Keller, 1996). The exchanger imports four Na^+ ions for each Ca^{2+} and K^+ ion extruded (forward mode of exchange; Cervetto et al., 1989; Schnetkamp et al., 1989; Rispoli et al., 1995), i.e., one net positive charge is imported per exchange cycle (Yau and Nakatani, 1984; McNaughton et al., 1986; Hodgkin et al., 1987). The cloning and sequencing of the retinal exchanger cDNA have been reported (Reiländer et al., 1992); however, most of the functional studies so far have been performed in vivo or on reconstituted systems. The exchanger cDNA (Reiländer et al., 1992) has been expressed in the baculovirus system, and the functional integrity of the purified protein was assessed in reconstituted liposomes. The work has used fluorescent probes to measure Na^+-dependent Ca^{2+} efflux, reporting a clear K^+ dependence of the reconstituted protein. In the work described here, the retinal Na^+:Ca^{2+},K^+ exchanger cDNA (Reiländer et al., 1992) was transiently expressed in 293 cells by transfection with plasmid DNA coprecipitated with Ca^{2+} phosphate. Only the reverse mode of the exchanger activity was recorded in whole-cell voltage-clamp experiments, as it proved impossible to record the activity in the forward exchanger mode because of the lethal effect of intracellular perfusion with high Ca^{2+}. The work has shown that the activity of the expressed protein was independent of K_o^+. Control whole-cell voltage-clamp experiments performed under the same experimental conditions on isolated rod outer segments (OSs) (Rispoli and Detwiler, 1991; Rispoli et al., 1993; Perry and McNaughton, 1993; Navangione et al., 1995, 1996) yielded the expected K_o^+ dependence.

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MATERIALS AND METHODS

Construction of expression vector pcNCKE

The 3.9-kb cDNA encoding the Na^+:Ca^{2+},K^+ exchanger from bovine rods (Reiländer et al., 1992) was isolated from BluescriptII KS and transferred to the eucaryotic expression vector pcDNAI as follows. The Bluescript construct was linearized by restriction cleavage with *Kpn*I that cut at the 5′ end of the cDNA insert, and was then treated with the Klenow enzyme to create blunt ends. The 5′ end of the exchanger DNA was then cleaved with *Not*I. The insert was ligated in the pcDNAI with the *Not*I site and the filled-in *Xba*I site.

Cell culture and transfection

Human embrionic kidney cells (293, ATCC, CRL 1573) were grown in minimum essential medium (Gibco BRL, Life Technologies; Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum (Sigma). For transfection, 2.5×10^5 cells were plated on coverslips in 35-mm-diameter Corning plastic dishes. Twenty-four hours after plating, the cells were washed twice and the dishes refilled with fresh medium. After 3 h of incubation at 5% CO_2, the cells were transfected by the DNA Ca^{2+} phosphate coprecipitation procedure (Chen and Okayama, 1987), using 3 μg DNA/dish. After 18 h of incubation at 3% CO_2, the cells were washed twice to remove the precipitate, the dishes were refilled with fresh medium, and the incubation was continued for an additional 24 h at 5% CO_2 to allow expression of plasmid DNA.

Immunocytochemistry

Cells were plated on coverslips in 35-mm-diameter dishes and transfected as described above. They were fixed in 3% formaldehyde, permeabilized with 0.1% Triton X-100, and incubated with the antibody anti-Na^+:Ca^{2+},K^+ exchanger (Haase et al., 1990) at 1:10 dilution in blocking buffer

containing 5% fetal bovine serum, 0.1% bovine serum albumin (BSA), 5% glycerol, and 0.04% NaN_3 in phosphate-buffered saline (PBS). The immunoreaction was revealed by an anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate used at 20 μg/ml (Boeringer, Mannheim, Germany).

Ca^{2+} uptake measurements

Cells transfected on a 10-cm-diameter dish were used for each series of experiments. They were washed twice with 1 ml of Na^+ loading buffer containing 160 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM ouabain, 2 mM $MgCl_2$, and 25 μM nystatin and incubated in the Na^+ loading buffer for 10 min at 37°C. Cells were then washed three times with 1 ml of 160 mM NaCl, 20 mM HEPES, 1 mM ouabain, and detached in 1 ml of this buffer by gently pipetting. After low-speed centrifugation, cells were resuspended in ~30 μl of the same buffer. They were then diluted ~30-fold in 275 μl of uptake buffer containing NaCl, KCl, or choline (160 mM); 20 mM HEPES (pH 7.4); 1 mM ouabain; 25 μM free Ca^{2+} (0.5 mM $CaCl_2$ and 0.475 mM EGTA), 1 μCi^{45} Ca^{2+}; and 1 μM valinomycin (included to offset the electrogenicity of the transport process; Caroni et al., 1980). A 50-μl aliquot was taken at each time point, placed on a Millipore membrane (0.45 μm pore diameter), vacuum filtered, and immediately washed three times with 0.5 ml of ice-cold stop buffer containing 160 mM KCl, 20 mM HEPES (pH 7.4), and 1 mM EGTA. The filters were then counted in a liquid scintillation counter (further details in Gabellini et al., 1996).

Voltage clamp

Preparation outer segments (OSs) were mechanically isolated from rods of the nocturnal lizard *Gekko gecko* (Rettili srl, Varese, Italy). Eyes were removed from dark-adapted animals that had been previously decapitated and pithed. The methods are described in detail elsewhere (Rispoli et al., 1993, 1995). The OSs were transferred to the recording chamber made of Teflon sandwiched between two glass microscope coverslips; the bottom one was coated with chloro-tri-*n*-butyl-silane to prevent the OS from sticking (Rispoli et al., 1993). The piece of glass coverslip containing the 293 cells was gently placed on the chamber bottom. OS and 293 cells were viewed with an infrared-sensitive CCD camera (XC-77CE, illumination wavelength >850 nm; Sony, Tokyo, Japan) coupled to a modified inverted microscope (IMT-2; Olympus, Tokyo, Japan) equipped with Hoffmann optics (Modulation Optics, Greenvale, NY). All experiments were performed in room light at room temperature ($T \approx 19$–24°C).

Solutions and recording procedure

Electrical recordings were carried out using the "whole-cell" configuration of the patch recording technique. Pipettes were prepared from 100-μl Drummond glass capillaries in the conventional manner (Hamill et al., 1981) and fire polished to a resistance of 7–11 MΩ. They were filled with one of the "intracellular" solutions indicated in the text with the subscript i. The 0 Ca^{2+} solutions were buffered with 2 mM EGTA unless specified otherwise; all solutions were buffered to pH ~7.4 with HEPES and NaOH, KOH, or LiOH, depending upon whether the solution contained high Na^+, high K^+, or 0 Na^+ and 0 K^+, respectively. The osmolality of the solutions that contained nonisotonic concentrations of the cations transported by the exchanger was adjusted to the Ringer osmolality with LiCl or choline chloride and checked with a microosmometer (13/13 DR; Roebling, Berlin, Germany). ATP (1 mM), CsCl (5 mM), and tetraethylammonium chloride (TEACl) (20 mM) were incorporated in all intracellular solutions used for the 293 cells; the last two chemicals were used to block the endogenous conductances. Ouabain (1 mM) was incorporated in all external solutions of the cells to abolish the current generated by the activity of the Na^+:K^+ pump. All chemicals were purchased from Sigma and Aldrich Chemicals (Milwaukee, WI). The perfusion solution was applied and removed by a peristaltic pump (Minipuls 2; Gilson, Villiers le Bel, France) connected to two drawn-down syringes inserted into the chamber. It proved impossible to perform fast solution changes (Rispoli et al., 1995), because the 293 cells were firmly attached to the glass and it was not possible to lift them to reach the perfusion pipette mouth. Some control experiments on the OSs were then performed with the peristaltic pump perfusion. Current was recorded with an Axopatch 1D amplifier (Axon Instruments, Burlingame, CA). Holding potential was 0 mV unless specified otherwise. In the recordings from both OS and 293 cells, the seal resistance ranged between 10 and 40 GΩ, the access resistance between 15 and 40 MΩ, and the input resistance between 1 and 10 GΩ.

Data analysis

Data were digitized in PCM format and recorded on DAT tapes, using a DTR-1801 tape recorder (Biologic, Claix, France), or were digitized, after filtering at half the sampling frequency, using an eight-pole Butterworth filter (VBF/8 Kemo; Beckenham, England), on-line or off-line by a TL-1 DMA interface (Axon Instruments), and stored on erasable magnetooptical disks (M2511A; Fujitsu, Tokyo, Japan). The figures were prepared using a commercial plotting program (Sigmaplot; Jandel Scientific, San Rafael, CA). The values in the text and figures are given as means ± SEM.

RESULTS

Protein expression and targeting

The retinal exchanger cDNA expressed in 293 cells, as described in Materials and Methods, was analyzed by immunological methods using a polyclonal antibody against the retinal exchanger (Haase et al., 1990). The results are shown in Fig. 1: the antibody clearly stained a population of cells (~20%) that corresponded approximately to the average number of transfected cells (one of which is shown in Fig. 1 *A*). This estimate agrees fairly well with the percentage of transfected cells expressing the active retinal exchanger, as estimated in a large number of electrophysiological recordings (~100; see below). The staining in nontransfected cells was much fainter (Fig. 1 *B*). Most of the immunoreactivity was visible in the plasma membrane (Fig. 1 *A*), indicating that the targeting of the expressed protein was correct. The expressed protein was also analyzed by Western blotting of transfected 293 cells. The antibody clearly recognized a polypeptide of ~160 kDa, which was absent in untransfected cells (data not shown). The reasons for the difference in molecular mass with respect to the exchanger protein in retina plasma membrane (apparent molecular mass 230 kDa; Haase et al., 1990) are very likely related to the extensive glycosylation of the protein in the retina (Schwarzer et al., 1997). The 293 cells probably glycosylate the exchanger only incompletely; the molecular mass of the expressed protein, as predicted from the cDNA, was expected to be ~130 kDa.

Exchange activity in transfected cells

Recordings of untransfected 293 cells in the presence of 5 mM Cs_i^+, 20 mM TEA_i^+, and 1 mM external ouabain did not show voltage-dependent or time-dependent currents (Fig. 2 *A*, 2 *B*, 2 *C*, *trace b*, and Fig. 3, *trace b*). Under these conditions, the current reversal potential was ~0 mV, irrespective of the ionic composition of the intracellular and

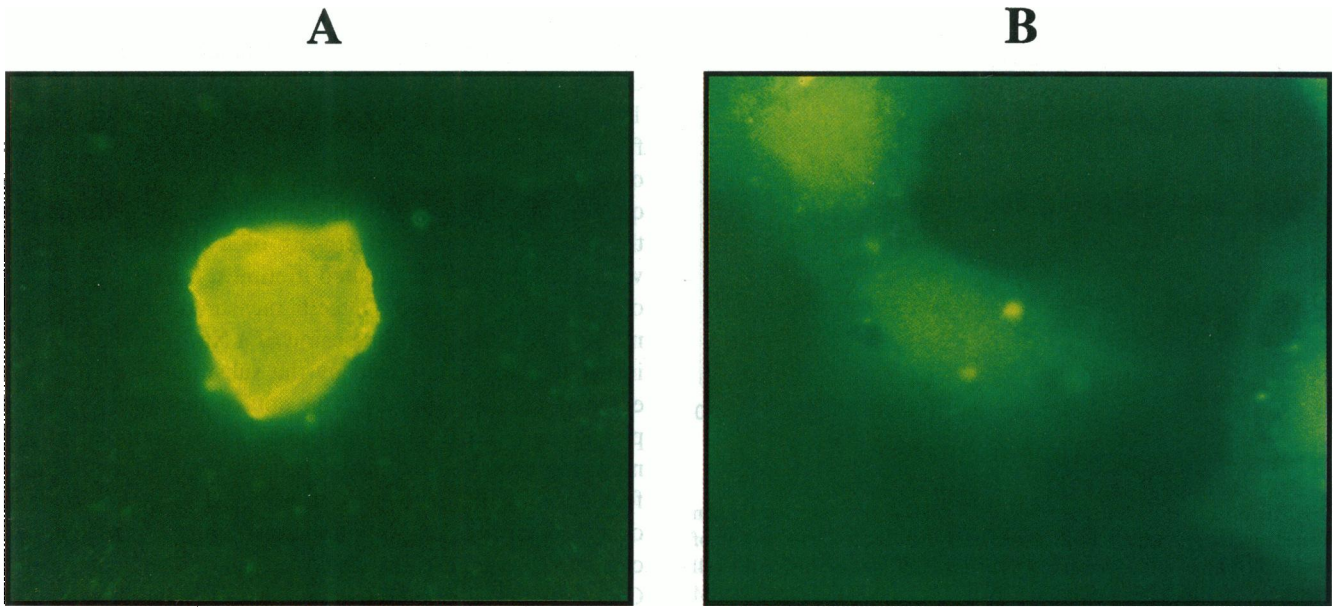

FIGURE 1 Immunostaining of a 293 cell expressing the retinal exchanger. (*A*) Immunostaining of a transfected 293 cell showing most of the exchanger immunoreactivity localized in the plasma membrane. (*B*) Nontransfected cells. Magnification: 1500×. Details are found in Materials and Methods.

extracellular solutions, and the input resistance, in the absence of Ca_i^{2+}, was always larger than 1 GΩ. Attempts were made to record the maximum reverse exchange current, using 128 mM Na_i^+, 0 Ca_i^{2+} (2 mM EGTA), 20 mM K_o^+, and 1 mM Ca_o^{2+}. Under these conditions, ~85% of the recordings from cells transfected with the protocol described in Materials and Methods were similar to those from the untransfected cells (i.e., as in Fig. 2, *A* and *B*, and in *trace b* of Fig. 2 *C*). The remainder of the recordings showed an outward current (Fig. 2 *C*, *trace a*, and Fig. 3, *trace a*), which increased progressively, as the pipette solution equilibrated with the intracellular milieu after the breakthrough, to a steady-state amplitude of 11.7 ± 1.9 pA (16 cells). This amplitude was close to that recorded from OSs (i.e., 15.0 ± 1.4 pA, 12 OS; Rispoli et al., 1996) under similar ionic conditions. However, this similarity is probably accidental, as discussed below. The recordings from cells showing the outward current could rarely last more than ~25 min, because in experiments of longer duration, the cells exhibited the same pathological phenomenology (Fig. 2 *C*, *trace a*) observed in cells perfused with high Ca_i^{2+} (Fig. 2 *C*, *trace c*; see below). This indicates that, despite the presence of 2 mM EGTA in the pipette solution, when an outward current was recorded, Ca^{2+} accumulated in the intracellular milieu was sufficient to promote apoptosis. The outward current dropped to zero upon removing Ca_o^{2+} or raising Na_o^+ to a level that virtually abolished the Na^+ gradient (in the presence of a saturating Ca_o^{2+} concentration; Fig. 3, *trace a*), whereas in untransfected cells the current remained at zero level (Fig. 3, *trace b*) in response to the same solution changes. The current level was unaffected by the removal of K_o^+ in transfected (11.1 ± 2.7 pA, seven cells; Fig. 3, *trace a*) or untransfected (Fig. 3, *trace b*) cells.

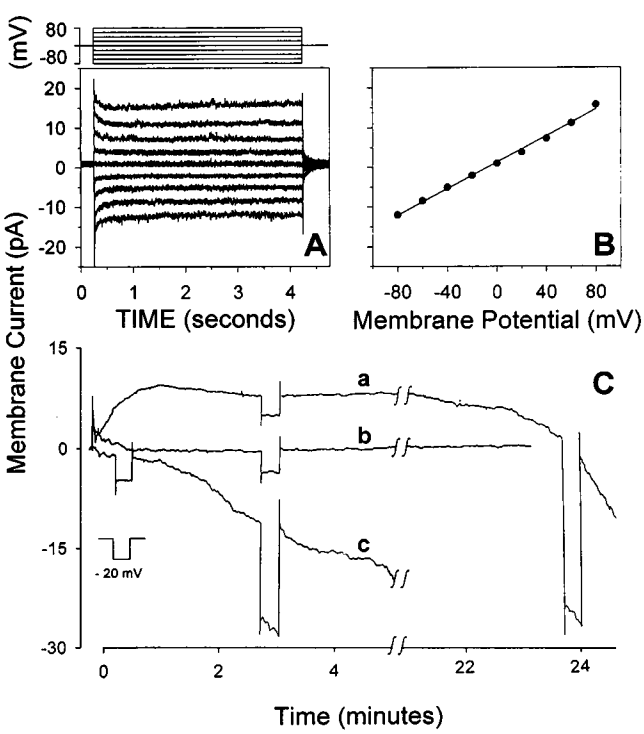

FIGURE 2 Comparison between recordings from untransfected and transfected cells. (*A*) An untrasfected 293 cell perfused intracellularly with Cs^+ and TEA^+ is stepped under voltage clamp from an holding potential of 0 mV to test pulse potentials ranging in 20-mV steps from −80 mV to +80 mV (current sampled at 500-μs intervals). (*B*) The steady-state current from each trace is plotted against the test potential; the resulting input resistance was ~6 GΩ. (*C*) Chart record of whole-cell current of *a*, a transfected cell in the presence of 128 mM Na_i^+, 0 Ca_i^{2+}, 20 mM K_o^+, and 1 mM Ca_o^{2+}; *b*, an untransfected cell in the same ionic conditions as *a*; *c*, an untransfected cell in the presence of 128 mM K_i^+, 50 μM Ca_i^{2+}, 153 mM Na_o^+, 0 Ca_o^{2+}. For all three traces, the breakthrough from cell attached to whole-cell recording occurred at time zero.

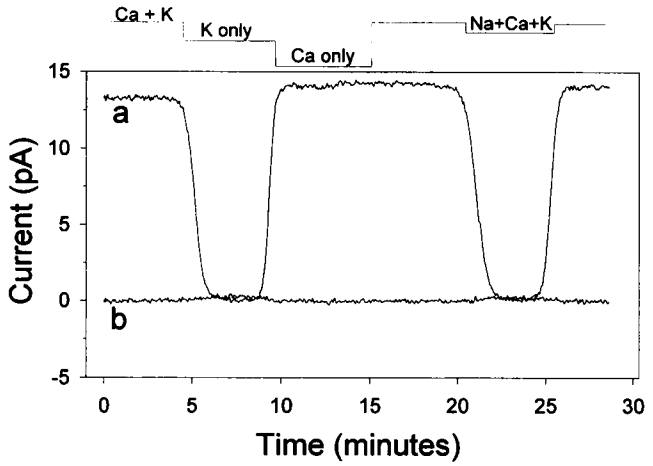

FIGURE 3 Effect of Ca_o^{2+} removal, K_o^+ removal, and Na_o^+ increase on reverse exchange current from transfected 293 cells. Chart record of whole-cell current in the presence of 128 mM Na_i^+ and perfusing the cell with either 20 mM K_o^+ + 1 mM Ca_o^{2+}, 20 mM K_o^+, 1 mM Ca_o^{2+}, or 130 mM Na_o^+ + 1 mM Ca_o^{2+} + 20 mM K_o. *Trace a*, Cell transfected with the exchanger; the current amplitude in 20 mM K_o^+ + 1 mM Ca_o^{2+} was 11.7 ± 1.9 pA (16 cells), whereas in 1 mM Ca_o^{2+} it was 11.1 ± 2.7 pA (7 cells); *trace b*, the same protocol as used in trace *a* applied to an untransfected cell.

These results are consistent with the view that the outward current recorded in ~15% of the transfected cells arose from the electrogenic activity of the exchanger operating in the reverse mode, in which Ca^{2+} is imported and Na^+ extruded. Importantly, however, the removal of K_o^+ completely suppressed the reverse exchange current recorded from OSs (Perry and McNaughton, 1993; Rispoli et al., 1995, 1996), whereas it had little if any effect on the current amplitude in 293 cells. On the other hand, the addition of 130 mM Na_o^+ to the 1 mM Ca^{2+} + 20 mM K^+ external solution had a minor effect on the reverse exchange current recorded from OSs (Rispoli et al., 1996), whereas it suppressed it in 293 cells. Even the similarity between the current amplitudes of the OS and the 293 cells must be taken with caution: the reverse exchange induces a large Ca_i^{2+} accumulation in OS because the disk stack prevents the efficient washout of Ca^{2+} by the patch pipette and thus causes a strong reduction in the steady-state amplitude of reverse exchange (Perry and McNaughton, 1993; Rispoli et al., 1996); the accumulation is expected to be much smaller in 293 cells. The aforementioned differences between the natural resident protein and that expressed from recombinant DNA could, in principle, be rationalized if the current generated by the latter (in Fig. 2 *C*, *trace a*, and Fig. 3, *trace a*) did not arise from the activity of an exchanger but from that of an ionic channel: i.e., the expressed protein would be a (yet unknown) Ca^{2+}-activated, Na^+-permeable channel. This possibility, however, was ruled out by the results of three independent tests. First, the current amplitude was similar to those shown in Fig. 2 *C* (*trace a*) and Fig. 3 (*trace a*), if the 120 mM Li_o^+ present in the Ca^{2+}-K^+ external solution was replaced by 120 mM external choline. This substitution would have been expected to increase Na^+ efflux through the hypothetical channel, because all Na^+ channels have a significant permeability to Li^+ (Hille, 1992). Second, the power spectrum of this current (Fig. 4) failed to reveal a Lorentzian component, but showed instead only the component proportional to the inverse of the frequency: this indicates that the current was not generated by the random opening and closing of a channel population with exponentially distributed lifetimes. Finally, the uptake of Ca^{2+} by transfected 293 cells measured with an isotopic method supported the conclusion of a Na^+-dependent Ca^{2+} influx that was unaffected by the substitution of K_o^+ with external choline (Fig. 5). The uptake reaction reached a plateau in ~30 s, at which time it corresponded to ~7 nmol/mg protein. No uptake was detected in cells transfected only with the expression vector. The apoptotic effect observed during long-duration recordings of the outward current also suggests that the expressed protein transported Ca^{2+}. In principle, the lack of K^+ regulation could be accounted for by a much higher affinity of the expressed exchanger for K^+, so that the K^+ contamination of the 0 K^+ solutions might have been sufficient to operate it. This seems unlikely, however, because atomic absorption spectroscopy measurements on the 0 K^+ solutions indicated a K^+ contamination lower than 3 μM (the apparent K_o^+ dissociation constant of the exchanger in the retina, during reverse operation, ranges from ~150 μM (in 0 Na_o^+) to ~7 mM (in 100 mM Na_o^+; Perry and McNaughton, 1993). The alternative possibility that the lack of K^+ regulation could be artifactually due to the interference of external ouabain with the K^+ transport site was also ruled out: ouabain (as well as Cs^+ and TEA^+) failed to affect the reverse exchange current amplitude recorded from OSs in the experimental conditions of Fig. 2 *C* (*traces a* and *b*) and Fig. 3. It can be

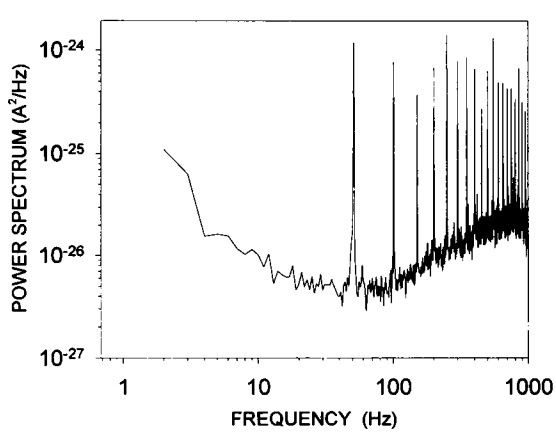

FIGURE 4 Power spectra of current fluctuations of the reverse exchange current. Data were sampled at 500 μs in 2000 point sweeps after the DC component was removed. The figure shows the average of 70 power spectra calculated from each sweep during reverse exchange operation. A similar power spectrum was recorded from the same cell in 0 Ca_o^{2+}, where the exchanger is not thought to be operational, indicating that the noise generated by the exchanger is below the resolution of these recordings. The spikes are 50 Hz line frequency noise and its harmonics.

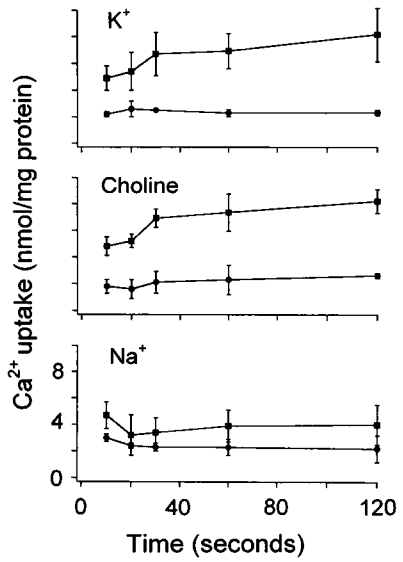

FIGURE 5 Isotopic measurement of the exchange activity. □, Uptake by cells expressing the retinal exchanger. ●, Uptake by cells transfected only with the vector (pcDNAI). (*Top*) Na^+-loaded cells diluted in the K^+ medium; (*middle*) diluted in the choline medium; (*bottom*) diluted in the Na^+ medium. The figure describes the results of four independent transfection experiments, each of them performed in duplicate. Vertical bars represent standard deviations. The details of the measurement are given in Materials and Methods.

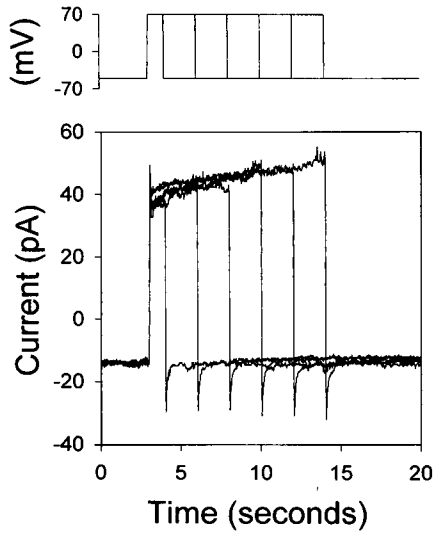

FIGURE 6 Forward and reverse modes of the retinal exchanger operation. The exchanger activity was studied exclusively with voltage pulses of progressively longer durations (1, 3, 5, 7, 9, and 11 s), in the presence of 40 mM Na_i^+, 10 mM K_i^+, 0.05 mM $EGTA_i$, 140 mM Na_o^+, 10 mM K_o^+, and 0.1 mM Ca_o^{2+}. Current was sampled at 500-μs intervals.

then concluded that, at least in the reverse mode of operation, the expressed exchanger does not operate in the Na^+:Ca^{2+},K^+ transport mode, but in the Na^+:Ca^{2+} mode only. It proved to be impossible to test the forward exchange activity in the transfected cells, as this would have required high Ca_i^{2+}. In the presence of Ca_i^{2+} (50 μM), an inward leak current developed (as shown by the progressive decrease in the input resistance; Fig. 2 *C*, *trace c*): most likely, cells entered apoptosis, as also indicated by their progressive budding. Attempts were made to record the exchange activity using the strategy described in Kofuji et al. (1992) and Gabellini et al. (1996). The first tests were performed on OSs (Fig. 3), but using 40 mM Na_i^+, 10 mM K_i^+, 0.05 mM $EGTA_i$, 140 mM Na_o^+, 10 mM K_o^+, and 0.1 mM Ca_o^{2+}, giving an exchanger reversal potential of about −60 mV. The OS was held at −60 mV, and the resulting current level was taken as the baseline. The potential was then brought to +70 mV to elicit reverse mode exchange. The potential was then returned to −60 mV, so that both the Na_i^+ depletion and the Ca_i^{2+} loading through the reverse mode of exchanger operation were expected to promote the forward mode of exchanger activation. The recorded current should reach an initial peak and then return to baseline, because of the extrusion of the Ca^{2+} load by the forward exchange operation and washed out by the patch pipette. However, the small "tail current" recorded was not affected by the duration of the depolarization period (i.e., by the size of the Ca_i^{2+} load and Na_i^+ depletion; Fig. 6) or by the removal of Ca_o^{2+}. Thus the above protocol was inadequate for the study of the forward exchange activity of the expressed protein.

DISCUSSION

The conclusion that the protein expressed in 293 cells transports Ca^{2+} in a Na^+-dependent but K^+-independent manner, as the cardiac exchanger does, is at variance with results on the exchanger of retinal photoreceptors. The similarity to the operation of the cardiac Na^+:Ca^{2+} exchanger was also supported by the fall in the exchange activity in response to the increase in Na_o^+, to the point where the Na^+ gradient was nearly abolished. This was observed in both electrophysiological recordings (Fig. 3)

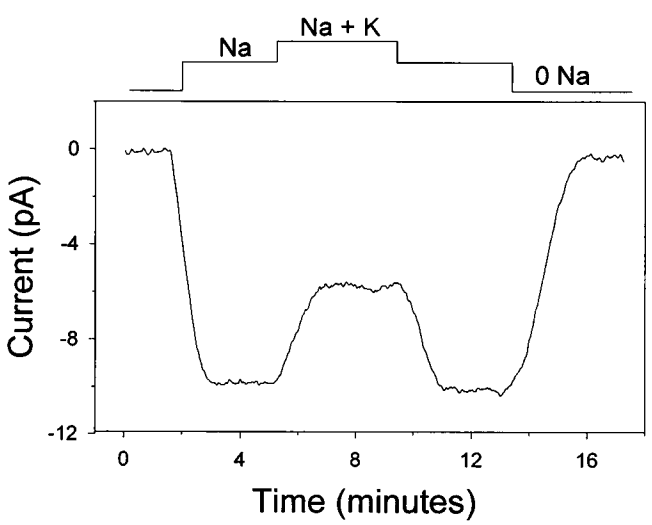

FIGURE 7 Chart record of the effect of K_o^+ rise on the exchange current recorded from an OS. K_o^+ was increased from 0 to 120 mM in the presence of 50 mM Na_o^+, 0 Ca_o^{2+}, 50 mM K_i^+, and 20 mM Ca_i^{2+}. The current amplitude in 0 K_o^+ was 11.4 ± 1.5 pA, whereas in 120 mM K_o^+ it was 6.8 ± 1.1 pA (five cells).

and in the Ca^{2+} uptake measurements (Fig. 4, *bottom*). This is typical of the cardiac-type Na^+:Ca^{2+} exchangers (Reeves and Sutko, 1983; Komuro et al., 1992; Noble et al., 1991), but not of the retinal exchanger (Perry and McNaughton, 1993; Navangione et al., 1995; Rispoli et al., 1995). On the basis of thermodynamic considerations, it is not expected that the current would fall to 0 upon raising Na_o^+: although the driving force for Na^+ is almost abolished, adequate energy is still stored in the K^+ and Ca^{2+} gradients, and a current can be recorded under these conditions in OSs. However, the Na^+:Ca^{2+} exchanger and the expressed retinal exchanger are not operative under the above conditions, indicating that the high Na^+ concentrations at the exchanger side where Na^+ is released induce their blockade. The different behavior of the two exchanger types could be rationalized by assuming that the apparent dissociation constant for Na_o^+ is larger for the natural retinal exchanger. This would predict that, upon raising Na_o^+, the release of Na^+ at the extracellular side of the "cardiac exchanger type" (operating in the reverse mode) would be impeded. Alternatively, the competition between Na^+ and Ca^{2+} at extracellular binding sites of the natural exchanger expressed in retina could be weaker than in the case of the "cardiac exchanger type." This would impede binding of Ca_o^{2+} in the presence of large Na_o^+ concentrations in the latter case, resulting in the exchanger blockade. On the other hand, the behavior of the expressed exchanger also showed similarities to that in the retinal cells, in that, at variance with other tissues (such as squid axon, squid optic nerve, guinea pig ventricular myocytes, and sarcolemmal vescicles; reviewed by DiPolo and Beaugé, 1991), it did not require Ca_i^{2+} for reverse exchange activation (Rispoli et al., 1995). Thus the behavior of the expressed exchanger was intermediate between the cardiac and the retinal types, i.e., under the recording conditions used, the expressed exchanger apparently switched from the Na^+:Ca^{2+},K^+ retinal mode to the Na^+:Ca^{2+} cardiac mode of operation. The lack of K^+ regulation of the expressed protein was also at variance with the large decrease in exchange activity (72%) observed with the purified exchanger, expressed in the baculovirus system, and reconstituted in lipid vesicles (Reiländer et al., 1992), upon increasing K_o^+ (from 25 to 100 mM) in the presence of 50 mM Na_o^+, 0 Ca_o^{2+}, and concentrations of K_i^+ and Ca_i^{2+} (50 mM and 4 mM, respectively) that would saturate the exchange activity. Such a large activity decrease was surprising, and indeed unexpected in the retinal exchanger under the above conditions, because the energy associated with K^+ transport, W_K, given by

$$W_K = R \cdot T \cdot \ln\left(\frac{[K^+]_i}{[K^+]_o}\right) + F \cdot V_m$$

is much smaller than the energy associated with the Na^+ transport, W_{Na},

$$W_{Na} = -4 \cdot \left(R \cdot T \cdot \ln\left(\frac{[Na^+]_i}{[Na^+]_o}\right) + F \cdot V_m\right)$$

and the Ca^{2+} transport, W_{Ca},

$$W_{Ca} = 2 \cdot R \cdot T \cdot \ln\left(\frac{[Ca^{2+}]_i}{[Ca^{2+}]_o}\right) + F \cdot V_m$$

where R, T, and F have their usual thermodynamic meanings and V_m is the membrane potential. In fact, W_{Na} is 2.3×10^4 cal · mol^{-1} and $W_{Ca} > W_{Na}$, whereas W_K goes from 1.7×10^3 cal · mol^{-1} to -1.7×10^3 cal · mol^{-1} when K_o^+ is raised from 25 to 100 mM (T = 298 K). Thus the change in K_o^+ in the experiments by Reiländer et al. (1992) would not have been expected to produce such a large variation in the exchanger activity. This conclusion was supported by whole-cell recordings from OSs, where it was necessary to increase K_o^+ from 0 to 120 mM (Fig. 7) to reach a 40% exchange current decrease in the presence of 50 mM Na_o^+, 0 Ca_o^{2+}, and saturating concentrations of K_i^+ and Ca_i^{2+} (50 mM and 20 mM, respectively; such a large Ca_i^{2+} concentration is necessary to keep a saturating level of Ca_i^{2+} in the vicinity of the exchanger; see Rispoli et al., 1995).

In principle, the difference in K^+ and Na^+ sensitivity between the exchanger expressed in 293 cells and that in the retina plasma membrane could have been due to a subtle misfolding of the expressed protein: a large-scale misfolding is very unlikely, given the correct delivery of a large part of the expressed protein to the plasma membrane. Minor sequence differencies, however, cannot be ruled out, and have already been shown to result in substantial conformational changes in the case of other proteins. Posttranscriptional modifications of the protein that occurred in the retina but not in 293 cells (e.g., glycosylation), could also rationalize the difference. The significantly lower molecular mass of the expressed protein would certainly be compatible with this explanation. The possibility of an accessory protein, present in the OS plasma membrane but not in that of 293 cells, which would confer K^+ sensitivity on the exchanger operation, could also be considered. This putative regulatory protein could be membrane bound, because no differences in exchanger properties were found between whole-cell and inside-out, excised patches from OSs (Rispoli et al., 1996). The modulation of ion transporter by an accessory protein (which may be an ion transporter as well) is not new; e.g., it has recently been found that the slowly activating K^+ current (I_{Ks}) of cardiac muscle is generated through the association of two membrane proteins, the K_V-LQT1 and the IsK (Berhanin et al., 1996, and Sanguinetti et al., 1996).

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