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SODIUM-EXTRUDING AND CALCIUM-EXTRUDING SODIUM/CALCIUM EXCHANGERS DISPLAY SIMILAR CALCIUM AFFINITIES

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Summary

Na^+/Ca^{2+} exchange activities in purely inside-out and mixed inside-out and right-side-out fish enterocyte basolateral plasma membrane vesicle preparations display equal affinities for Ca^{2+}, showing that only the intracellular Ca^{2+} transport site of the Na^+/Ca^{2+} exchanger is detected in experiments on vesicle preparations with mixed orientation. Therefore, Ca^{2+} pump and Na^+/Ca^{2+} exchange activity may be compared directly without correction for vesicle orientation. The Na^+/Ca^{2+} exchange activity in fish enterocyte vesicles is compared to the activity found in dog erythrocyte vesicles. The calcium-extruding exchanger in fish basolateral plasma membranes shows values of K_m and V_{max} for calcium similar to those found for the sodium-extruding exchanger in dog erythrocyte membranes, indicating that differences in electrochemical gradients underlie the difference in cellular function of the two exchangers.

Introduction

Na^+/Ca^{2+} exchange was first described in mammalian heart cells (Reuter and Seitz, 1968) and in squid giant axons (Baker *et al.* 1969). At first it was thought that this exchange mechanism was characteristic of excitable cells, in which countercurrents of Na^+ and Ca^{2+} underlie the electrical events associated with cell activity. In recent years, however, similar antiporters have been demonstrated in a number of non-excitable cell types (Taylor, 1989). In these cells the exchanger may serve a number of functions. Its role in sodium extrusion in sodium-transporting epithelia is well-established (Taylor, 1989). In canine erythrocytes, the Na^+/Ca^{2+} exchanger is involved in cell volume regulation and Na^+ homeostasis (Parker, 1987; Ortiz and Sjodin, 1984).

We have reported on Na^+/Ca^{2+} exchange in fish enterocytes (Flik *et al.* 1990). The Na^+/Ca^{2+} exchanger uses the transmembrane Na^+ gradient, which is generated by Na^+/K^+-ATPase activity, to extrude Ca^{2+} from the cytoplasm into the extracellular fluid. By so doing it makes an essential contribution to cellular

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Ca^{2+} homeostasis (Schoenmakers *et al.* 1992*a*). The dependence of net transepithelial Ca^{2+} uptake on the Na^+ gradient across the basolateral membrane emphasizes the functional importance of the exchanger in transepithelial Ca^{2+} uptake (Flik *et al.* 1990).

The function of the Na^+/Ca^{2+} exchanger in erythrocytes of dogs and ferrets is quite different. In these cells a powerful ATP-dependent Ca^{2+} pump produces a low intracellular Ca^{2+} concentration. The high intracellular Na^+ concentration of $150\,mmol\,l^{-1}$ (Flatman and Andrews, 1983) is, however, lower than that expected for a Nernstian distribution ($220\,mmol\,l^{-1}$; Milanick, 1991). Lacking Na^+/K^+-ATPase, these cells use a Na^+/Ca^{2+} exchanger, which harnesses the large inward Ca^{2+} gradient to extrude excess Na^+ (Parker, 1987).

What determines whether a Na^+/Ca^{2+} exchanger will perform Na^+ extrusion or Ca^{2+} extrusion? Electrochemical constraints dictate the zero-flux state and operating direction of the exchanger at given ionic conditions and membrane potential (Reeves, 1985), but Ca^{2+} affinity, especially of the intracellular site, may well be the main regulator of the velocity of the exchange process once non-zero-flux conditions apply. We tested whether a difference in Ca^{2+} affinity of the Na^+/Ca^{2+} exchanger is the basis for differences in cellular function in different cell types. To this end, we investigated the calcium affinities of Na^+/Ca^{2+} exchange proteins in plasma membrane preparations from fish enterocytes and dog erythrocytes. We have previously shown that fish enterocyte plasma membrane vesicles may be used to study Na^+/Ca^{2+} exchange not only in a mixture of right-side-out (ROV) and inside-out (IOV) vesicles, but also in a pure preparation of IOVs. The Na^+/K^+-ATPase activity in these membrane vesicles was used to generate a Na^+ gradient in IOVs, which allowed an assessment of the Ca^{2+} affinity of the intracellular Ca^{2+} site (Flik *et al.* 1990). The Ca^{2+} kinetics of the exchangers in these two preparations were similar. Also, the Ca^{2+} affinity observed in the fish IOVs was similar to that displayed by the ROV+IOV preparation. We conclude that the functioning of the Na^+/Ca^{2+} exchanger is dictated by the differences in cation concentrations between the inside and outside of the living cells.

Materials and methods

Fresh, heparinized blood was obtained from beagles at the Central Animal Laboratory (Nijmegen). Isolation of erythrocytes and preparation of plasma membrane vesicles were performed as described for human erythrocytes by Sarkadi *et al.* (1980) with two modifications. (1) β-Mercaptoethanol was not used in the isolation procedure since, as indicated by Parker (1987), we found an inhibitory effect of β-mercaptoethanol on Na^+/Ca^{2+} exchange activity (data not shown). (2) The two final washes with 10 volumes of $10\,mmol\,l^{-1}$ Tris–HCl (pH 7.4) described by Sarkadi *et al.* (1980) were replaced by a single final wash with 50 volumes of this buffer and centrifugation at $50\,000\,g_{av}$ for 20 min.

After isolation, the vesicles were frozen in liquid N_2 and stored at −70°C. Vesicles were used within 2 weeks of isolation; no decline in Na^+/Ca^{2+} exchange

activity was noted during this period. On the day of experimentation, the vesicle suspension was thawed, suspended in 40 volumes of assay medium by 15 passes through a 23-gauge needle, pelleted by centrifugation at 50 000 g_{av} for 20 min, and resuspended in an appropriate volume of assay medium. Depending on experimental requirements (see below), the assay medium consisted of either 150 mmol l^{-1} NaCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4, or 100 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4. The protein content of the membrane vesicle preparations was 1.8±0.5 g l^{-1} (mean±S.D., N=12), as determined with a commercial reagent kit (Bio-Rad) with bovine serum albumin (BSA) as a reference.

Fish (*Oreochromis mossambicus* Peters; hereafter called tilapia) enterocyte basolateral plasma membrane vesicles were prepared as previously described (Flik *et al.* 1990). Briefly, the intestine was flushed with ice-cold saline and cut lengthwise. Intestinal mucosa was collected by scraping off the epithelium on an ice-cooled glass plate. The mucosal cells were homogenized in an isotonic sucrose buffer, and nuclei and cellular debris were pelleted by centrifugation at 1400 g_{av} for 10 min. The supernatant was collected and centrifuged for 25 min at 150 000 g_{av}. The resulting pellet consisted of two parts: a firm brown part, containing around 90 % of the succinic acid dehydrogenase activity of the homogenate, and a white and fluffy layer, mainly consisting of plasma membranes. The latter was resuspended in an isotonic sucrose buffer and brought to 37 % (w/w) sucrose. On top of 8 ml of this suspension 4 ml of isotonic sucrose buffer was layered, and an isopycnic centrifugation was performed on the assembly for 90 min at 200 000 g_{av}. The membranes at the interface were collected using a 23-gauge needle, and mixed with 25 volumes of the final isotonic assay buffer (150 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4). After centrifugation at 180 000 g_{av} for 35 min, the pellet was rinsed twice with assay buffer and resuspended by 25 passages through a 23-gauge needle. The protein concentration of the vesicle suspensions was approximately 1 g l^{-1}.

Specific endo- and exoenzymes can be used to test the sidedness of vesicles in a membrane preparation. The activity of an exoenzyme measured in a preparation containing IOVs, ROVs and leaky vesicles (A_-) must originate from the ROVs and the leaky vesicles. After the addition of a small amount of detergent to permeabilize the IOVs, the enzyme activity in all the vesicles can be measured (A_+). The percentage of IOVs can then be calculated as $\%IOV = 100 \times (1 - A_-/A_+)$. A similar method using an endoenzyme will yield information on the percentage of ROVs. The percentage of IOVs in our preparations was determined on the basis of the acetylcholine esterase latency of the membrane preparation (Steck and Kant, 1974). In the dog erythrocyte membrane preparations, the latent activity of this exoenzyme unmasked by Triton X-100 (2 g l^{-1} at 1 g l^{-1} protein) indicated that 57±9 % (mean±S.D., N=9) of the vesicles were IOVs. The percentage of ROVs was determined on the basis of the glyceraldehyde-3-phosphate dehydrogenase latency of the membrane preparation (Steck and Kant, 1974). The latent activity of this endoenzyme when unmasked by

Triton X-100 indicated that 26±6 % (N=7) of the vesicles were ROVs. Therefore, the configuration of the dog erythrocyte plasma membrane preparation was 57 % IOVs, 26 % ROVs and 17 % leaky vesicles. The percentage of IOVs is similar to that reported for canine erythrocyte vesicles by Ortiz and Sjodin (1984). Thawing and resuspending the membrane vesicles did not alter membrane configuration (data not shown). The configuration of the tilapia enterocyte plasma membrane vesicle preparation was 29 % IOVs, 24 % ROVs and 47 % leaky fragments (Flik *et al.* 1990).

Na^+/Ca^{2+} exchange activity in plasma membrane vesicles from dog erythrocytes and fish enterocytes was assayed as previously described for the fish preparation (Flik *et al.* 1990). Briefly, 5 μl of membrane vesicles equilibrated with 150 mmol l^{-1} NaCl was added to 120 μl of medium containing either 150 mmol l^{-1} NaCl (blank) or 150 mmol l^{-1} KCl. The radioactive concentration of ^{45}Ca (specific activity 19 GBq mmol^{-1}) in the transport media was 1.3 MBq ml^{-1}. After 5 s at 37 °C, the reaction was stopped by the addition of 1 ml of ice-cold isotonic stopping solution containing 1 mmol l^{-1} $LaCl_3$, followed by filtration through a nitrocellulose filter (Schleicher & Schuell ME25). The difference in ^{45}Ca accumulation was taken to represent Na^+-gradient driven Ca^{2+} transport. To test whether there is a significant build-up of membrane potential as a result of the electrogenic behaviour of the Na^+/Ca^{2+} exchanger (Flik *et al.* 1990; Reeves, 1985), we measured Na^+/Ca^{2+} exchange activity as follows: membrane vesicles, equilibrated in 100 mmol l^{-1} NaCl + 50 mmol l^{-1} KCl, were transferred either to 100 mmol l^{-1} NaCl + 50 mmol l^{-1} KCl (blank) or to 100 mmol l^{-1} LiCl + 50 mmol l^{-1} KCl. The difference in the amount of ^{45}Ca accumulated was assumed to result from Na^+/Ca^{2+} exchange activity. Addition of 5 μg ml^{-1} of the K^+ ionophore valinomycin (dissolved in ethanol) was used to short-circuit vesicle membrane potential. Lithium has previously been shown not to activate Na^+/Ca^{2+} exchange in dog erythrocytes (Ortiz and Sjodin, 1984; Parker, 1988). It mimics the effects of K^+ on Na^+/Ca^{2+} exchange in other systems (Mullins and Requena, 1989). Pilot experiments with fish enterocytes and dog erythrocytes ascertained that the substitution of 100 mmol l^{-1} KCl by 100 mmol l^{-1} LiCl did not have a significant effect on Na^+/Ca^{2+} exchange velocity (data not shown).

Na^+/Ca^{2+} exchange activity in fish enterocyte IOVs was tested with an assay procedure utilizing the ouabain-sensitive Na^+/K^+-ATPase activity of the fish membranes to load IOVs selectively with Na^+(Flik *et al.* 1990). Vesicles were resuspended in 150 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4. A sample of the vesicles was resuspended in assay medium containing 1 mmol l^{-1} ouabain. After preincubation on ice for 2 h to allow diffusion of ouabain into the intravesicular space of the IOVs, where the ouabain binding site of the Na^+/K^+-ATPase is exposed, ^{45}Ca uptake at 37 °C was measured in a medium consisting of 135 mmol l^{-1} KCl, 15 mmol l^{-1} NaCl, 0.8 mmol l^{-1} Mg^{2+}, 3 mmol l^{-1} ATP and 20 mmol l^{-1} Hepes/Tris, pH 7.4. The reaction was stopped by an eightfold dilution in ice-cold isotonic stopping buffer containing 1 mmol l^{-1} $LaCl_3$. Vesicle-associated ^{45}Ca in media containing 1 mmol l^{-1} ouabain represented ATP-

dependent ^{45}Ca transport and passive diffusion of ^{45}Ca. The extra ^{45}Ca taken up in the absence of $1\,mmol\,l^{-1}$ ouabain was assumed to result from Na^+/Ca^{2+} exchange activated by Na^+, which was introduced intravesicularly by the action of the Na^+/K^+-ATPase during the assay period. We used this procedure to analyze the Ca^{2+}-dependence of the cytosol-oriented Ca^{2+} site of the Na^+/Ca^{2+} exchanger. The Ca^{2+} concentrations of the media in all assays described above were varied from 1.5×10^{-7} to $2\times10^{-5}\,mol\,l^{-1}$ using metal-cation-chelating agents to create accurately known free Ca^{2+} concentrations (Schoenmakers *et al.* 1992*b*).

Data points were obtained in duplicate. Non-linear regression data analysis of the mean values of a number of experiments (N values given below) testing the entire kinetic curve of initial transport velocities as a function of Ca^{2+} concentration yielded estimates of K_m and V_{max} for calcium. The standard deviations of the values obtained in this way cannot, however, be used for further statistical testing.

Results and discussion

We investigated whether a build-up of membrane potential occurred in the dog erythrocyte membrane vesicles during the assay period (i.e. 5 s). Although there is no direct evidence for electrogenicity of Na^+/Ca^{2+} exchange in dog erythrocytes, as opposed to fish enterocytes (Flik *et al.* 1990), the cooperative dependency of exchange activity on Na^+ (Parker, 1988) suggests that the Na^+/Ca^{2+} exchanger also carries a current in these cells (Sarkadi and Parker, 1991). A significant build-up of vesicle membrane potential due to the electrogenic behaviour of the Na^+/Ca^{2+} exchanger can easily influence the outcome of kinetic experiments with this carrier (Reeves, 1985). Omission of the K^+ ionophore valinomycin ($5\,\mu g\,ml^{-1}$) from the K^+/valinomycin clamp medium should allow the build-up of an inhibitory potential. However, no such effects were observed: in the absence of valinomycin in a medium containing $18\,\mu mol\,l^{-1}$ Ca^{2+} no significant change in Na^+/Ca^{2+} exchange activity was observed [exchange velocity was $7.7\pm1.6\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$ ($N=5$) in the absence of valinomycin and $7.9\pm1.0\,nmol\,min^{-}\,mg^{-1}$ ($N=7$) in its presence]. We ascribe the apparent lack of a significant build-up of membrane potential during the 5 s of our assay to the relatively low Na^+/Ca^{2+} exchange velocities ($13\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$, see below) compared to velocities observed for Na^+/Ca^{2+} exchange in membrane preparations from excitable cells, such as sarcolemmal membranes ($120\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$; Reeves, 1985).

We then analyzed the Ca^{2+} dependence of the initial velocities of Na^+/Ca^{2+} exchange in dog erythrocyte plasma membrane vesicles. Na^+/Ca^{2+} exchange displayed a V_{max} of $12.7\pm0.8\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$ and a K_m of $3.7\pm0.4\,\mu mol\,l^{-1}$ Ca^{2+} ($N=11$). The fish plasma membrane preparation, containing a mixture of IOVs and ROVs, displayed a Na^+/Ca^{2+} exchange activity with a V_{max} of $14.3\pm0.7\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$ and a K_m of $1.1\pm0.1\,\mu mol\,l^{-1}\,Ca^{2+}$ ($N=9$). When the Ca^{2+} dependency of the Na^+/Ca^{2+} exchanger in IOVs was tested, a V_{max} of $1.7\pm0.2\,nmol\,Ca^{2+}\,min^{-1}\,mg^{-1}$ and a K_m of $1.8\pm0.2\,\mu mol\,l^{-1}$ were found ($N=5$;

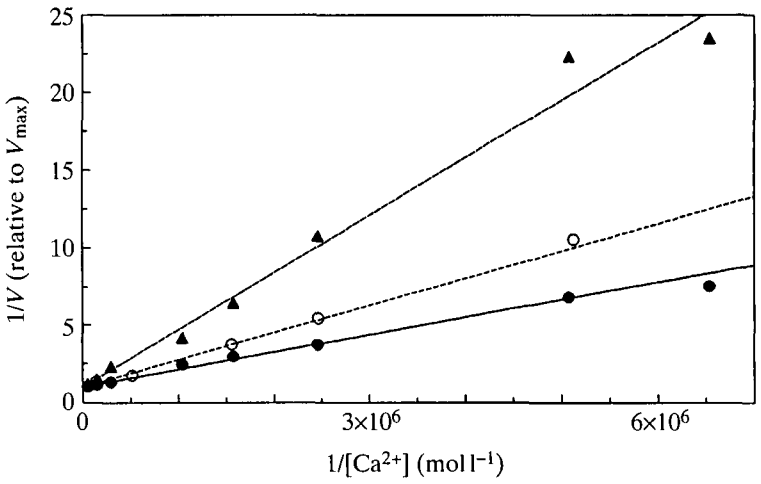

Fig. 1. Lineweaver–Burk plot of initial velocities of Na^+/Ca^{2+} exchange as a function of free Ca^{2+} concentration in three membrane vesicle preparations. Filled circles and the solid line indicate the mixed inside-out and right-side-out preparation from basolateral enterocyte membranes of the tilapia (N=9), while the open circles indicate the Na^+/Ca^{2+} exchange activity of the population of inside-out vesicles (N=5). Filled triangles denote Na^+/Ca^{2+} exchange activity in the dog erythrocyte membrane preparation (N=11). Data points were obtained in duplicate per experiment. Data were normalized to the calculated V_{max} value.

Fig. 1). The lower V_{max} is caused by the smaller Na^+ gradient in this experimental arrangement: using the Na^+ dependency of the Na^+/Ca^{2+} exchanger published previously (Flik *et al.* 1990) to calculate the intravesicular [Na^+] created through Na^+/K^+-ATPase activity, we obtain a concentration of only 17 mmol l^{-1}.

These experiments do not indicate a difference in K_m of several orders of magnitude for the intra- and extracellular site of the Na^+/Ca^{2+} exchanger, as reported for whole cells. If there is a difference between the values shown here, it may well have resulted from the longer assay period (i.e. 60 s) necessary for detection of the low exchange activity in the IOV preparation. We therefore conclude that the Ca^{2+} site in IOVs displays the same Ca^{2+} affinity as the Ca^{2+} sites in a mixture of IOVs and ROVs. Three causes may be advanced for this phenomenon. First, the Na^+/Ca^{2+} exchanger may be functionally symmetrical with respect to Ca^{2+} on both sides of the plasma membrane. However, numerous data obtained from whole cells contradict this hypothesis (Reeves, 1985). Second, the asymmetry reported in whole cells may be lost during vesicle isolation. Both of these explanations have been suggested before (Philipson, 1985). A third, more likely, possibility seems to have been overlooked so far: Li *et al.* (1991) showed that the low specific radioactivity of media with high Ca^{2+} concentrations impedes detection of the low-affinity extracellular Ca^{2+} site in vesicle experiments. They also showed that Na^+/Ca^{2+} exchange of a mixed population of IOVs and ROVs could be completely inhibited by a synthetic peptide that binds specifically to the

cytosolic side of the protein, demonstrating that all the measured Na^+/Ca^{2+} exchange activity in the preparation occurred in the IOVs. Therefore, we propose that the kinetic data on Ca^{2+} dependency of Na^+/Ca^{2+} exchange in the mixed vesicle preparations from tilapia enterocytes and dog erythrocytes represent the Ca^{2+} dependency of the *intracellular* Ca^{2+} site of the exchanger.

Na^+/Ca^{2+} exchange activity in erythrocytes of dogs and related species has been previously investigated in whole cells (Altamirano and Beaugé, 1985; Milanick, 1989; Parker, 1987, 1988) as well as in membrane vesicles (Ortiz and Sjodin, 1984). Estimates of Ca^{2+} affinity are scarce (Milanick, 1989; Parker, 1988) and concern only the extracellular Ca^{2+} site of the exchanger. As stated above, the vesicle assay yields a K_m value for the intracellular Ca^{2+} site. Owing to competition for the transport site by the high intracellular Na^+ concentration, the low level of intracellular Ca^{2+} hardly activates the dog erythrocyte Na^+/Ca^{2+} exchanger. The high gradient for Ca^{2+} entry drives the extrusion of Na^+ *via* the exchanger. Na^+/Ca^{2+} exchange activity is primarily controlled by the intracellular Na^+ concentration, in line with its proposed physiological function in these cells (Parker, 1987).

The intracellular Na^+ concentration in tilapia enterocytes is much lower than in dog erythrocytes, with total cell Na^+ amounting to $87\pm14\,mmol\,l^{-1}$ cell water (van der Velden, 1990). Free sodium ions (their concentration is estimated at $9\,mmol\,l^{-1}$; Flik *et al.* 1990) will hardly compete with intracellular calcium ions for their common site. This, combined with a more negative membrane potential ($-60\,mV$; Bakker and Groot, 1988) than that found in erythrocytes ($-10\,mV$), ensures the involvement of the Na^+/Ca^{2+} exchanger in Ca^{2+} extrusion across the basolateral membrane. The importance of the exchanger for Ca^{2+} homeostasis in tilapia enterocytes is illustrated when we consider the maximal velocities of the two processes responsible for Ca^{2+} extrusion in these cells: the ATP-dependent pump attains a V_{max} of $0.63\pm0.04\,nmol\,min^{-1}\,mg^{-1}$ (Flik *et al.* 1990), but the Na^+/Ca^{2+} exchanger displays a V_{max} of $14.3\pm0.7\,nmol\,min^{-1}\,mg^{-1}$. We now know that these figures are directly comparable, since both represent activities of the respective membrane proteins in the IOVs only. In Fig. 2, we illustrate the relative importance of the ATP-dependent Ca^{2+} pump and the Na^+/Ca^{2+} exchanger in Ca^{2+} extrusion from the tilapia enterocyte by plotting the Ca^{2+} dependencies of both mechanisms. Although the K_m of the Na^+/Ca^{2+} exchanger appears to be rather high for a Ca^{2+} extrusion mechanism, the high capacity of the exchanger more than compensates for this. In these cells, the inward Na^+ gradient and the inside-negative membrane potential drive Ca^{2+} extrusion *via* Na^+/Ca^{2+} exchange. The velocity of this process is directly dependent on the intracellular $[Ca^{2+}]$, since the exchanger will not be saturated by intracellular Na^+ (Flik *et al.* 1990).

Thus, while dog erythrocytes have a powerful ATP-dependent Ca^{2+} pump to ensure their Ca^{2+} homeostasis, tilapia enterocytes need the Na^+/Ca^{2+} exchanger to extrude excess Ca^{2+}. Conversely, tilapia enterocytes exhibit a potent Na^+/K^+-ATPase activity in their basolateral membranes, with which they efficiently

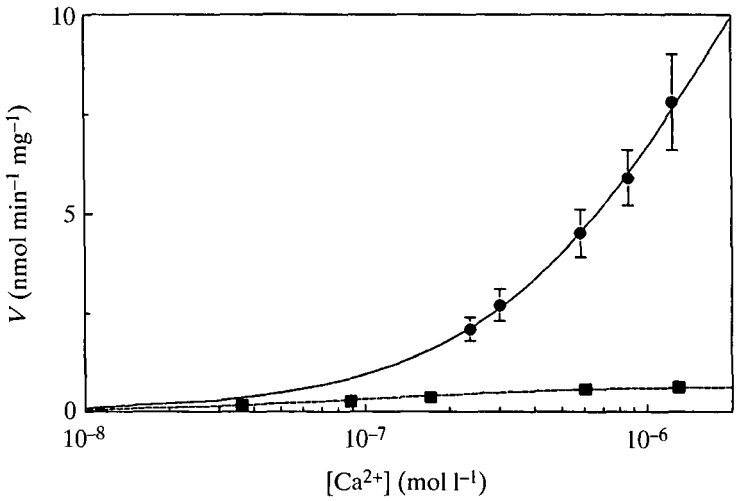

Fig. 2. A comparison of the ATP-dependent Ca^{2+} pump and the Na^+/Ca^{2+} exchanger in basolateral plasma membrane vesicles from tilapia enterocytes. The filled circles and solid curve denote the Na^+/Ca^{2+} exchanger, and the filled squares and dashed curve represent the ATP-dependent Ca^{2+} pump. Values are mean±S.D. for five experiments. Deviations were not drawn when they were smaller than the symbol size. The Na^+/Ca^{2+} exchange activity exceeds the activity of the ATP-dependent Ca^{2+} pump threefold at free Ca^{2+} concentrations around 100 nmol l^{-1}.

extrude Na^+. Dog erythrocytes, lacking this mechanism, use the Na^+/Ca^{2+} exchanger to extrude Na^+. Our study of the kinetic parameters of these two exchange proteins shows that these vastly different functions can be performed by proteins displaying very similar characteristics. Differences in ionic and electrical driving forces, resulting from cell specialisation and cell energy status, are reflected in the carrier's change of function.

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