$Na^+ - Ca^{2+}$ Exchange in Plasma Membranes of Crayfish Striated Muscle

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Abstract. $Na^+ - Ca^{2+}$ exchange rates and some physico-chemical properties of the exchanger were studied in crayfish striated muscle membranes enriched in plasma membranes prepared by differential centrifugation of muscle microsomal fraction on discontinuous sucrose density gradient. The lightest subfraction with the highest Na^+, $K^+ - ATPase$ and $Mg^{2+} - ATPase$ activities also showed the highest $Na^+ - Ca^{2+}$ exchange rates. A number of physico-chemical characteristics of the $Na^+ - Ca^{2+}$ exchanger found in the present experiments were similar to those reported for excitable membranes of mammals, except for the temperature optimum (20°C for the crayfish).

Key words: $Na^+ - Ca^{2+}$ exchange — Crayfish striated muscle — Plasma membranes

Introduction

$Na^+ - Ca^{2+}$ exchange is present in the plasma membranes of excitable (Gilbert and Meissner 1982; Langer 1982; Orlický et al. 1983) and non-excitable tissues (Bernstein and Santacana 1985; Famulski and Carafoli 1982; Jayakamur et al. 1984), with the highest specific activities found in excitable tissues. There are numerous data concerning $Na^+ - Ca^{2+}$ exchange in the plasma membranes of the heart muscle and nerve tissue. The exchanger from these tissues could be reconstituted into proteoliposomes (Schellenberg and Swanson 1982; Wakabayashi and Goshima 1982) and/or arteficial lipidic membranes, and partially purified (Barzilai et al. 1984; Soldati et al. 1985). The physico-chemical properties of the $Na^+ - Ca^{2+}$ exchanger found in the plasma membranes of rabbit striated muscle were identical with those described in the heart and nerve tissue, the former showing however significantly lower specific activity (Gilbert and Meissner 1982). Our experiments were performed on striated muscle of the crayfish (*Astacus fluviatilis*) which, in contrast to rabbit muscle, operates on a

pure calcium electrogenesis principle (Zachar 1981); a high $Na^+ - Ca^{2+}$ exchange activity (Šajter et al. 1978) has been suggested in this tissue. Since the crayfish is a poikilotherm it seemed also interesting to compare the physicochemical properties of the exchanger from the crayfish muscle with those of the exchanger from homoiotherms.

Materials and Methods

Reagents: Tris-base, sucrose, Na_4ATP(Merck), sodium dodecyl sulfate, EGTA(Serva), imidazol-(Fluka), pronase(Sigma), Verapamil(Orion), $^{45}CaCl_2$(Institute of Isotopes of the Hungarian Academy of Sciences); all other chemicals of analytical grade were purchased from Lachema Brno.

Preparation of crayfish striated muscle plasma membranes: Striated muscles excised from the tail were homogenized with 6 × 30 s strokes in a Polytron homogenizer (Luzern, Switzerland) at 800 rpm in a medium of the following final composition (in mmol . l^{-1}): sucrose 300, Tris-HCl 20 pH 7.4, EGTA 5; the tissue to medium ratio was 1:5 (w/v). The homogenate was centrifuged for 20 min at 12,000 × *g* in a MSE 17 centrifuge. The supernatant was decanted, the sediment rehomogenized 1:3 and centrifuged as above. Pooled supernatants were filtered through several layers of cheesecloth and subsequently centrifuged at 100,000 × *g* in a VAC 60 ultracentrifuge. The sediment obtained was stirred in 0.6 mol . l^{-1} KCl solution buffered to pH 7 with 20 mmol . l^{-1} Tris-HCl, and after 1 h the suspension was centrifuged for 1 h at 100,000 × *g*. The sediment was suspended in 0.3 mol . l^{-1} sucrose and layered on a discontinuous sucrose density gradient (7 ml of each concentration), consisting of 0.6; 0.8; 1.0; and 1.2 mol . l^{-1} sucrose buffered to pH 7.4 with 20 mmol . l^{-1} Tris-HCl. The specimens were centrifuged for 6 h at 100,000 × *g* using a Beckman SW 28 ultracentrifuge rotor. The sediments obtained at the limits of gradients were sucked off into Pasteur pipettes, diluted with 3 volumes of 160 mmol . l^{-1} Tris-HCl, pH 7.4 and centrifuged for 1 h at 140,000 × *g*. The sediments obtained were stirred in 160 mmol . l^{-1} NaCl solution buffered to pH 7.4 or to increasing pH with 20 mmol . l^{-1} Tris or imidazol-HCl (see legend to Figure 4). One ml of the medium contained 1 mg of membrane protein. In one series of experiments the membranes were pretreated with pronase in w/w ratios 100:1, 1,000:1, 2,000:1 for 15 min, then quickly cooled on ice and centrifuged for 1 h at 140,000 × *g*.

When sufficient information could be obtained, only experiments with crude microsomal fractions were carried out.

$Na^+ - Ca^{2+}$ exchange in the membranes was estimated by the rapid filtration method through GF/C glass fibre filters (Schellenberg and Swanson 1982) at an initial external Ca^{2+} concentration of 20 μmol . l^{-1} (except for determination of calcium concentration dependence), Mg^{2+} – and Na^+, K^+ – ATPase activities were determined according to Lau et al. (1977), and the proteins according to Lowry et al. (1951) after preceding solubilization of membrane proteins in 2% SDS solution. $Na^+ - Ca^{2+}$ exchange rates are expressed in nmol . mg^{-1} prot . min^{-1}, ATPase activities in μmol P_i . mg^{-1} prot . h^{-1}.

Results

As can be seen from the diagram in Table 1, the microsomal fraction of the crayfish striated muscle could be separated into 5 subfractions (a — e) according

to the buoyant density. With regard to the protein contents and to $Na^+ - Ca^{2+}$ exchange rates, the highest total and specific activities were found in the lightest subfraction "a". The specific activity of the subfraction sedimenting at the $0.6 - 0.8\,mol \cdot l^{-1}$ sucrose limit was close to that of the original microsomal fraction while the total $Na^+ - Ca^{2+}$ exchange activity was similar to that obtained in the lightest subfraction. In three remaining subfractions of higher buoyant densities the $Na^+ - Ca^{2+}$ exchange was only 1/10 of the recovered activity. Mg^{2+} – and $Na^+ + K^+$ –stimulated ATPase activities had distribution patterns very similar to that of $Na^+ - Ca^{2+}$ exchange (Table 1). Active $Na^+ - Ca^{2+}$ countertransport could be confirmed in experiments in which A23187 added into the incubation medium after 45 s of $Na^+ - Ca^{2+}$exchange process released the intravesicularly accumulated calcium (Fig. 1).

$Na^+ - Ca^{2+}$ exchange rates in membrane preparations with the highest activities were dependent on the extravesicular calcium concentration and followed a first-order kinetics. Graphical analysis according to Lineweaver-Burk of the results obtained revealed a K_m value of $19\,\mu mol \cdot l^{-1}$ and V_{max} of $120\,\mu mol \cdot mg^{-1}$ prot.min^{-1} (Fig. 2). $Na^+ - Ca^{2+}$ exchange was decreasing with increasing extravesicular sodium (Na_o); a half-maximal inhibition was observed at Na_0 of approx. $20\,mmol \cdot l^{-1}$ and the exchange reaction stopped at $100\,mmol \cdot l^{-1}$ Na_o (Fig. 3). $Na^+ - Ca^{2+}$ exchange increased with increasing pH, the highest values being obtained in alkaline milieu at pH 8 (Fig. 4). By increasing the temperature of the incubation medium a distinct temperature optimum was found at 20° C (Fig. 5).

Verapamil added to membrane vesicles 5 min before $Na^+ - Ca^{2+}$ exchange measurements inhibited Na^+ dependent calcium accumulation; verapamil itself did not influence passive calcium binding to the membranes. The inhibitory effect of verapamil was concentration dependent, and was relatively higher in membranes partially digested with pronase in a ratio of 1,000:1 (Fig. 6). At this condition, approx. 20 % of the proteins were split off the membrane and the $Na^+ - Ca^{2+}$ exchange activity was only 30 % of control values. Incubation of the membranes with 100:1 pronase liberated 40 % of membrane proteins and the $Na^+ - Ca^{2+}$ exchange activity was completely lost. Polyacrylamide-gel SDS electrophoresis revealed the loss of high molecular weight proteins and accumulation of polypeptides below M_r 30,000 (results not shown).

SH blockers *p*-chlormercuribenzoate (PCMB) and dithionitrobenzene (DTNB) ($1\,mmol \cdot l^{-1}$) almost completely inhibited the $Na^+ - Ca^{2+}$ exchange. When $1\,mmol \cdot l^{-1}$ dithiothreitol was added for 5 min to the incubation medium after 5 min incubation with the SH blockers, the $Na^+ - Ca^{2+}$ exchange activity was partially restored in the presence of PCMB, but not with DTNB. In the presence of PCMB $Na^+ - Ca^{2+}$ exchange decreased from the control values of 23 nmol $^{45}Ca^{2+} \cdot mg^{-1}$ prot $\cdot min^{-1}$ to 1.2 nmol; the addition of dithiothreitol was

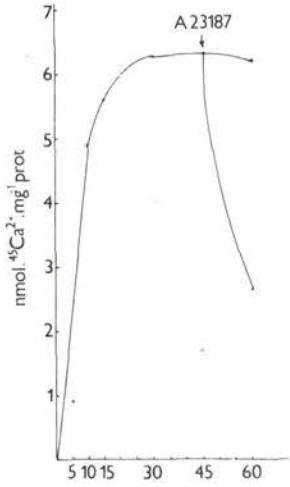

Fig. 1. Time dependent $^{45}Ca^{2+}$ accumulation in subfraction "a" (see Table 1). Arrow indicates the addition of the ionophore A 23187. Each point represents the mean value of 3 measurements.

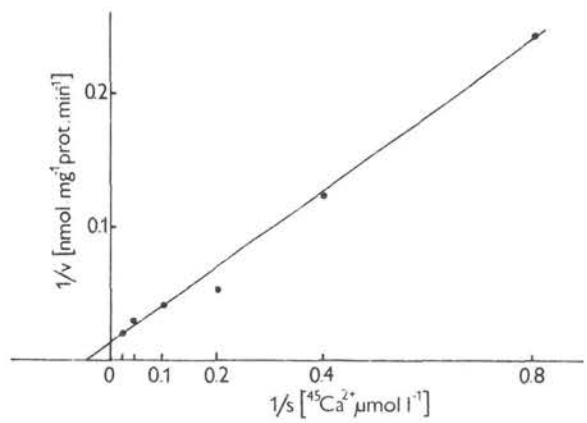

Fig. 2. Lineweaver-Burk's plots (reciprocal values of velocities against reciprocal values of Ca^{2+} concentrations) in subfraction "a" (see Table 1). Each point represents the mean of 4 measurements.

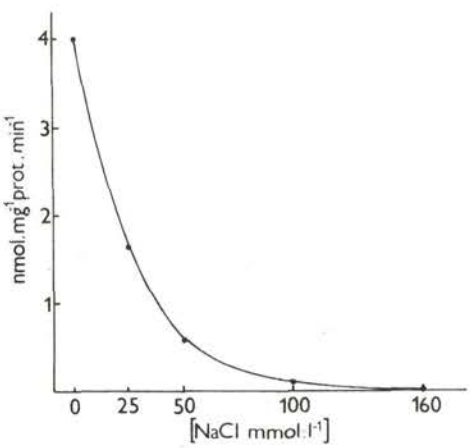

Fig. 3. Inhibition of $Na^+ - Ca^{2+}$ exchange in crude microsomal fraction of crayfish striated muscles by increasing Na^+ ion concentrations in the external medium. Mean values of 3 measurements.

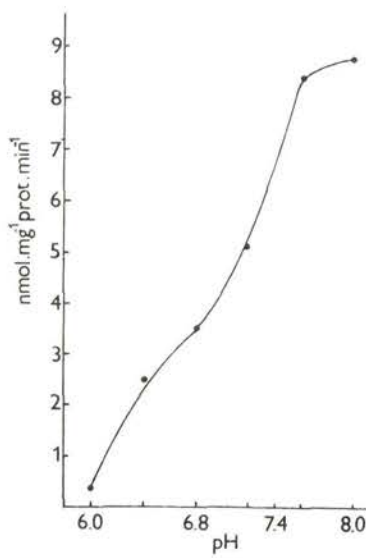

Fig. 4. pH dependence of $Na^+ - Ca^{2+}$ exchange in crude microsomal fraction of crayfish striated muscles; 50 mmol.l^{-1} imidazol-HCl buffer.

Table 1. Na^+–Ca^{2+} exchange in nmol . mg^{-1} prot. min^{-1}, Mg^{2+} – ATPase, $Na^+ + K^+$ – ATPase activities in μmol P_i . mg^{-1} prot . h^{-1} and protein content in mg in the crayfish striated muscle microsomal membrane fraction (Mic) and in its 5 subfractions separated after 6 h centrifugation at 100,000 × *g* on discontinuous sucrose density gradient 0.3—0.6 (*a*), 0.6—0.8 (*b*), 0.8—1.0 (*c*), 1.0—1.2 (*d*); sediment in 1.2 mol . l^{-1} sucrose (*e*). Mean values of 3 experiments. S. E. M. ranged between 6—9 % of the mean. For details see section Materials and Methods.

Fractions	$Na^+ - Ca^{2+}$ exchange	% of total	Mg^{2+} – – ATPase	% of total	$Na^+ + K^+$ – – ATPase	% of total	Proteins	% of total
Mic	7.6	100	3.7	100	5.4	100	28.7	100
a	19	40	8.7	37.6	13.3	39.4	4.6	16.1
b	8.3	38	4.2	40	7.9	51.4	10.1	35.2
c	3.7	5.9	2.7	9	5.3	12	3.5	12.2
d	2.0	1.7	1.8	3	0.9	1	1.8	6.2
e	0	0	1.7	2.5	0	0	1.6	5.5
Recovery %		85.6		92.1		103.8		75.3

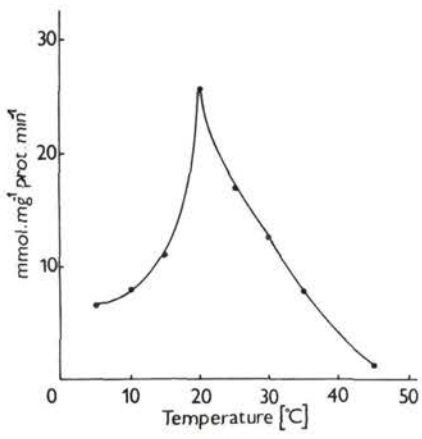

Fig. 5. Temperature dependence of $Na^+ - Ca^{2+}$ exchange in subfraction "a" (see Table 1). Each point is the mean of 6 measurements.

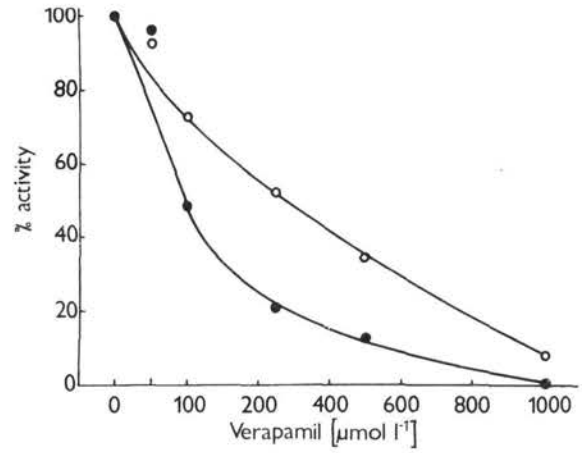

Fig. 6. Verapamil inhibition of $Na^+ - Ca^{2+}$ exchange in native plasma membranes (○—○), fraction "a" from Table 1 and in the membranes pretreated with pronase 100:1 (●—●). Each point represents mean value of 6 measurements. For details see section Materials and Methods.

followed by an increase in the $Na^+ - Ca^{2+}$ exchange rate to 12.7 nmol $^{45}Ca^{2+} \cdot mg^{-1}$ prot. min^{-1} (mean of 2 independent experiments).

ATP present in the incubation medium at 2 mmol. l^{-1}, either alone or in combination with 10 μmol. l^{-1} c-AMP, did not influence the $Na^+ - Ca^{2+}$ exchange rates in crayfish striated muscle plasma membranes.

Discussion

In our experiments a subfraction of crayfish striated muscle microsomes enriched in plasma membrane markers $Na^+ + K^+ -$ ATPase and $Mg^{2+} -$ ATPase (Lau et al. 1977) showed an $Na^+ - Ca^{2+}$ exchange rate 2.5 times higher as compared with the original microsomal fraction (Table 1); in subfractions of higher buoyant densities containing predominantly sarcoplasmic reticulum membranes (Tomková et al. 1984), the exchange was negligible. In rabbit striated muscle the $Na^+ - Ca^{2+}$ exchange lower by one order of magnitude (Gilbert and Meissner 1982, Bottlíková — personal communication) is obviously located in the sarcolemmal and not the tubular membranes (Brandt et al. 1980; Gilbert and Meissner 1982). Our results are consistent with the results of

other authors; however, they do not rule out that $Na^+ - Ca^{2+}$ exchange might also be present in the crayfish tubular membranes. In our experiment the highest $Na^+ - Ca^{2+}$ exchange activity was observed in that subfraction in which T-tubule membranes occurred (Tomková et al. 1984). This subfraction was prepared according to a method similar to that of Rosemblatt et al. (1984) for rabbit tubular system preparation, and the enzymatic properties of this subfraction were very similar to those reported for rabbit T-tubular membranes (Kirley and Schwartz 1984). The surface area of tubular membranes of crayfish striated muscle fibre is about 5 times larger than that of the plasma membrane (Uhrík et al. 1980) and it is through this membrane that calcium enters the crayfish muscle fibre which operates on the solely calcium electrogenesis principle (Zachar 1981). Calcium entering the crayfish muscle fibre leaves the transmembrane sodium gradient unchanged; it cannot therefore be excluded that transmembrane sodium gradient mediates calcium efflux in the tubular membranes of the crayfish muscle fibre. If $Na^+ - Ca^{2+}$ exchange occurred in the crayfish sarcolemma only, then, with regard to the total area of membrane structures in the muscle fibre (Uhrík et al. 1980), $Na^+ - Ca^{2+}$ exchange rates in the crayfish sarcolemma should be much higher than those obtained in the present experiments. A high $Na^+ - Ca^{2+}$ exchange in the crayfish muscle fibre plasma membrane has already been suggested by Šajter et al. in 1978. Nevertheless, the crayfish striated muscle with a high specific $Na^+ - Ca^{2+}$ exchange activity seems to be a good marker for the crayfish striated muscle plasma membranes; also, it is a tissue suitable for purification of the exchanger.

The K_m value for calcium in the crayfish muscle plasma membrane was 19 μmol . l^{-1} (Fig. 2). Very similar values have been found for rabbit striated muscle membrane (Gilbert and Meissner 1982), heart sarcolemma (Ledvora and Hegyvary 1983; Phillipson and Nishimoto 1982), nerve tissue membranes (Gill - et al. 1981; Schellenberg and Swanson 1982) and for smooth muscle membranes (Morel and Godfraind 1984).

$Na^+ - Ca^{2+}$ exchange decreased with increasing extravesicular sodium in the incubation medium (Fig. 3), with the half-maximal inhibition reached at about 20 mmol . $^{-1}$ Na_0, a value very close to those reported by others (Gilbert and Meissner 1982; Murphy et al. 1986; Phillipson 1985; Reeves and Sutko 1983).

The pH optimum for $Na^+ - Ca^{2+}$ exchange in crayfish muscle plasma membranes was slightly alkaline (pH 8) (Fig. 4). This value is identical with that reported for rabbit muscle plasma membrane (Gilbert and Meissner 1982), myocardial sarcolemma (Phillipson et al. 1982) or nerve tissue plasma membranes (Ruščák et al. 1985).

The temperature optimum (Fig. 5) for $Na^+ - Ca^{2+}$ exchange in mammals is at 37°C (Orlický et al. 1983; Schellenberg and Swanson 1982) which corresponds to the physiological value of body temperature. Considering that crayfish

is a poikilotherm, the optimum temperature of 20°C observed for the plasma membranes of crayfish muscle can be considered as physiological for this species.

Verapamil inhibited $Na^+ - Ca^{2+}$ exchange in the crayfish muscle plasma membranes in a dose-dependent manner (Fig. 6), with half-maximal inhibition at 10^{-4} mol/l. This value is very close to that found for nerve tissue membranes (Erdreich et al. 1983; Liron et al. 1985). Since verapamil did not influence calcium binding to the membranes its inhibitory action was likely due to changes in membrane fluidity as a result of its lipophilic nature (Pang and Sperelakis 1983). This is accentuated by the results obtained with pronase digested membranes. Under conditions of pronase digestion, large molecular weight membrane proteins are split off the membrane, with the access of verapamil to membrane phospholipids being facilitated; the inhibitory effect may thus become more pronounced at lower concentrations similarly as in the nerve tissue membranes (Juhász et al. 1985). In contrast to myocardial sarcolemma (Phillipson and Nishimoto 1982) only a decrease of $Na^+ - Ca^{2+}$ exchange was observed following partial digestion of crayfish plasma membrane with pronase.

Blockade of free SH groups of membrane proteins induces changes in the protein tertiary structure, a loss of the channel functions (Goll et al. 1984) and a loss of $Na^+ - Ca^{2+}$ exchange (Orlický et al. 1985) in the nerve tissue plasma membrane. Disappearance of $Na^+ - Ca^{2+}$ exchange was also observed in the crayfish muscle plasma membranes. The inhibitory effect of PCMB could partially be reversed in the crayfish muscle plasma membranes similarly as it could be in brain plasma membranes (Orlický et al. 1985).

$Na^+ - Ca^{2+}$ exchange in myocardial sarcolemma has been suggested to be regulated by phosphorylating/dephosphorylating reactions catalyzed by endogenous membrane kinases and phosphatases dependent on calcium and calmodulin, but not c-AMP (Carafoli et al. 1984). This conclusion could not be confirmed in the crayfish muscle plasma membranes. In accordance with our previous results obtained in brain plasma membranes (Orlický et al. 1986) we could not observe any effect of membrane protein phosphorylation on $Na^+ - Ca^{2+}$ exchange rates.

In conclusion, it can be stated that the $Na^+ - Ca^{2+}$ exchanger present in the crayfish striated muscle plasma membranes has similar physico-chemical properties as that in excitable mammalian tissues, except for the temperature optimum. Due to relatively high $Na^+ - Ca^{2+}$ exchange rates this system seems to be a suitable marker for plasma membranes of the crayfish striated muscles. Because its activity remains unchanged during one week of storage at 2–4°C, crayfish striated muscle plasma membranes will be used to reconstitute and purify the exchanger.

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