Na^+—Ca^{2+} Exchange in Rat Brain Microsomal Membranes Pretreated with Pronase and/or SDS

M. Ruščák, J. Orlický, O. Juhász and J. Zachar

Centre of Physiological Sciences, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Czechoslovakia

Abstract. The effects of pronase and/or SDS pretreatment on Na^+—Ca^{2+} exchange were studied in rat brain microsomal membranes. Pronase in concentrations that liberated 11 % of the membrane proteins stimulated the Na^+—Ca^{2+} exchange. When about 24 % of the proteins were split off, the results did not differ from those in control experiments. When 40 % or more of the proteins were solubilized, Na^+—Ca^{2+} exchange was abolished. Pronase pretreatment did not change the K_m value for Ca^{2+}, it increased V_{max} only. The effect of pronase was partially blocked by Trasylol. Neuraminidase had no effect on Na^+—Ca^{2+} exchange. SDS pretreatment of the membranes inhibited Na^+—Ca^{2+} exchange: when 25 % of membrane proteins were solubilized with SDS, the Na^+—Ca^{2+} exchange was abolished while the same amount of proteins split off with pronase did not change the rate of Na^+—Ca^{2+} exchange as related to membrane proteins. Ischaemia lasting for 2—4 h or complete hypoxia which should stimulate endogenous proteinases due to the rise of free intracellular calcium did not influence the Na^+—Ca^{2+} exchange. A decrease in Na^+—Ca^{2+} exchange rate was observed when proteins with molecular weights between 45,000 and 20,000 were split off from the membranes. It is assumed that the Na^+—Ca^{2+} antiporter is a polypeptide from the group of proteins within the above molecular weights.

Key words: Na^+—Ca^{2+} exchange — Rat brain microsomes — Pronase digestion — SDS extraction — Membrane proteins

Introduction

A great number of data are available concerning the Na^+—Ca^{2+} countertransport in excitable tissues; however, the molecular basis of this system still remains unknown. An inherent component of the Na^+—Ca^{2+} exchange mechanism seem to be the proteins. The involvement of proteins has been proved in experiments in

Presented at the 2nd International Symposium on Ionic Channels and Membrane Receptors, 21—26 May 1984, Smolenice, Czechoslovakia

which Na^+—Ca^{2+} countertransport systems from brain and heart muscle membranes solubilized with detergents could be reconstituted into artificial lipidic vesicles, giving equal or higher specific activities than in native membranes (Schellenberg and Swanson 1982; Wakabayashi and Goshima 1982; Luciani 1984). Pretreatment of sarcolemmal membranes with proteinases stimulated the Na^+—Ca^{2+} exchange system (Phillipson and Nishimoto 1982). Our present experiments also support the view that proteins are involved in the Na^+—Ca^{2+} exchange system: mild proteolysis of rat brain microsomal membranes with pronase stimulated, while a severe one inhibited the Na^+—Ca^{2+} exchange rate. On the other hand, extraction of membranes with SDS always inhibited the transport system.

Materials and Methods

Materials: sucrose (Merck); β-mercaptoethanol, EGTA, EDTA, Tris, dalton standards MS II (Serva); Trasylol (Bayer); neuraminidase (Sigma); pronase (Koch-Light); bovine serum albumin (Mann Res. Lab.); acrylamid, N,N'bisacrylamid (Serva); $^{45}CaCl_2$ activity 40 MBq.ml^{-1} (Institute of Isotopes of the Hungarian Academy of Sciences); other reagents of analytical grade were from Lachema, Brno; Millipor filters glass microfibre CG/C (Whatman).

The microsomal membrane fraction was prepared from the rat brain cortex. The tissue was excised in a cooled room and homogenized in a glass homogenizer (1 g of tissue per 8 ml of the homogenizing medium of the following final composition in mmol.l^{-1}: sucrose 320; EDTA 5; EGTA 10; β-mercaptoethanol 2; pH adjusted to 7.4 with 2.5 mol.l^{-1} NaOH solution). The homogenates were centrifuged for 30 min at 12,000 × *g* in a MSE 17 centrifuge. After decanting the supernatants the sediments were rehomogenized (1:3) and centrifuged under the same conditions as above. The combined supernatants were centrifuged for 1 h at 53,000 × *g* in a VAC 602 ultracentrifuge. The sediment of microsomal membranes was stirred in 20 ml of 5 mmol.l^{-1} sodium phosphate or Tris-HCl buffers, pH 7.4, and centrifuged at 53,000 × *g* for 1 h. The final sediments were dissolved in sodium phosphate or Tris-HCl buffer solutions, pH 7.4, giving 1 mg of protein per 1 ml. One part of suspended membranes was immediately taken for the estimations of Na^+—Ca^{2+} exchange. The remaining suspensions were divided into 7 aliquots and treated further as follows: the control tube was incubated for 15 min at 25 °C, the remaining tubes were incubated for the same interval at 25 °C with various amounts of pronase and/or SDS as given in detail in the legends to the figures. The specimens were then either immediately taken for Ca^{2+} uptake measurements or cooled on ice and centrifuged for 1 h at 100,000 × *g*. The supernatants were decanted, the sediments stirred in 20 mmol.l^{-1} Tris-HCl, pH 7.4, and both fractions were taken for protein determinations.

For Ca^{2+} uptake assays the microsomal membrane vesicles were incubated for 4—12 h at 2—4 °C in 160 mmol.l^{-1} NaCl + 5 mmol.l^{-1} sodium phosphate or 20 mmol.l^{-1} Tris-HCl buffers, pH 7.4, to allow sodium equilibration in the vesicles (Schellenberg and Swanson 1981). Uptake assays were carried out by adding 50 µl of Na^+ loaded vesicles, containing 20—40 µg of proteins, for 15 s into 1 ml of 160 mmol.l^{-1} KCl + 20 mmol.l^{-1} Tris-HCl, pH 7.4, or 1 ml of 160 mmol.l^{-1} NaCl buffered with Tris-HCl (blanks), containing various amounts of $^{45}Ca^{2+}$ as indicated in the legends to the figures. The uptake was stopped after 15 s by rapid filtration through a millipore glass fibre filter. Dried filters were suspended in SLT 41 Spolana solution and the radioactivity was counted in a Tricarb 3320 scintillation counter.

PAA gel electrophoresis of membrane bound proteins was carried out according to Laemli (1970).

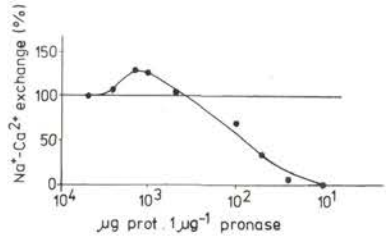

Fig. 1. Na^+—Ca^{2+} exchange rates in rat brain microsomal membranes pretreated for 15 min at 25 °C with increasing amounts of pronase expressed as percentual value of controls (100 % = 9.6 nmol.mg^{-1} prot.min^{-1}. Each point represents mean values of 3 independent experiments. The specimens were used for measurements without further centrifugation. Ca^{2+} in the medium = 10 µmol.l^{-1}.

In all results presented herein, blank values were subtracted from the values obtained in KCl media.

Proteins were determined according to Lowry et al. (1951) with a preceeding solubilization of membranes with 0.5 % SDS solution. Bovine serum albumin was used as a standard.

Results were statistically processed using Student's *t*-test.

Results

It could be shown that pronase, in relation to the amount of membrane proteins, stimulated or inhibited the Na^+—Ca^{2+} cotransport in rat brain cortex microsomal membranes. Maximal stimulation was observed when the membranes were incubated for 15 min at 25 °C in a protein-to-pronase weight ratio of 1000 : 1. At a ratio of 100 : 1 or less, the Na^+—Ca^{2+} exchange was depressed (Fig. 1). The effects of pronase on calcium transport could be prevented by adding Trasylol into the medium in amounts 5—25 times higher than that of pronase present in the medium: in membranes incubated with pronase in a ratio 10 : 1 Na^+—Ca^{2+} exchange practically disappeared, while in the presence of Trasylol in a ratio to pronase of 25 : 1, 36 % of the activity was still preserved as compared with the control experiments. Neuraminidase in weight ratios to proteins of 1 : 50—1 : 10 did not change the Na^+—Ca^{2+} exchange rates.

Since the above results relate to proteins present in the medium prior to the pronase treatment and not to those actually bound to the membranes after the digestion with pronase, in further experiments the transport velocities in membranes sedimented for 1 h at 100,000 × *g* after pronase pretreatment were investigated. In these experiments, the sodium-dependent calcium transport was enhanced in membranes pretreated with small amounts of pronase, and decreased after the digestion with higher concentrations of pronase. At a pronase to protein ratio of 1 : 1000, 11 % of the proteins were solubilized, at a ratio of 1 : 100, 24 % and at a ratio of 1 : 10, 40 % of the membrane proteins were found in the

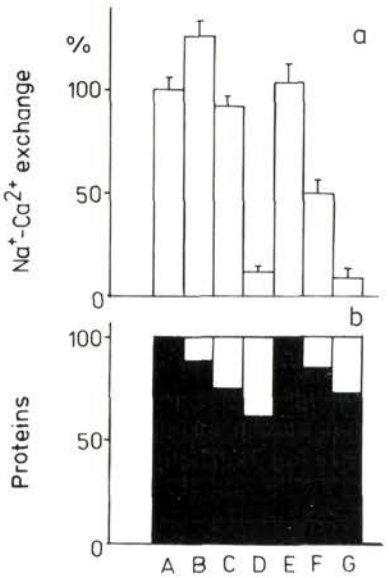

Fig. 2. *a*) Na^+—Ca^{2+} exchange in rat brain microsomal membranes pretreated with pronase and/or SDS for 15 min at 25 °C and recentrifuged at 100,000 × *g* for 1 h. Activity expressed as percentual value of controls (100 % = 9.8 ± 0.7 nmol.mg^{-1} prot.min^{-1}). Initial Ca^{2+} concentration in the medium 10 μmol.l^{-1}. *b*) Proteins in the supernatants (empty columns) and in the sediments (full columns) of microsomal membranes pretreated with pronase and/or SDS. *A* — control values. Pronase pretreatment: *B* — in the weight ratio to proteins 1:10^3, *C* — 1:10^2, *D* — 1:10^1. SDS pretreatment: *E* — in the weight ratio to proteins 1:10, *F* — 1:4, *G* — 1:2. For details see Materials and Methods.

supernatants. Pretreatment of the membranes with increasing amounts of SDS significantly inhibited the Na^+—Ca^{2+} exchange: when about 1/4 of the membrane proteins was solubilized the Na^+—Ca^{2+} countertransport disappeared, while equal amounts of proteins liberated with pronase were without any effect on calcium transport (for summarized results see Fig. 2).

Bilateral ligature of both carotid arteries, spreading depression elicited at ligated carotid arteries or total tissue hypoxia after decapitation, lasting for 2—4 h, did not influence the rates of Na^+—Ca^{2+} exchange in rat brain microsomal membrane vesicles.

SDS polyacrylamide gel electrophoresis of pronase-pretreated membrane polypeptides revealed that the enzyme first attacked polypeptides with a high molecular weight. The disappearance of large polypeptides had, however, no influence on the rate of Na^+—Ca^{2+} exchange. Only a decrease in the content of polypeptides with molecular weights between 45,000 and 20,000 (Fig. 3) resulted in a diminished velocity of Na^+—Ca^{2+} exchange (Fig. 2). Similar conclusions could be drawn from the results obtained on SDS-pretreated membranes. Contrary to

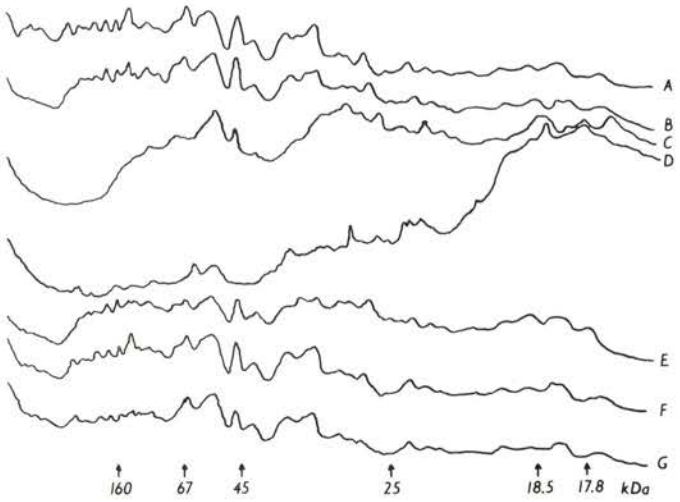

Fig. 3. Densitograms of electrophoreograms of rat brain microsomal membranes pretreated with pronase and/or SDS for 15 min at 25 °C and recentrifuged at 100,000 × *g* for 1 h. Symbols *A*—*G* as in Fig. 2. Standard kits used ($Mr \times 10^3$): myoglobin 17.8, chymotrypsinogen 25, albumin (egg) 45, albumin (bovine serum) 67, aldolase 160.

pronase SDS was more active on polypeptides with molecular weights below 45,000 (Fig. 3), and at the same time, it strongly inhibited the Na^+—Ca^{2+} exchange (Fig. 2). Thus, it seems probable that a polypeptide with a molecular weight between 45,000 and 20,000 is involved in the Na^+—Ca^{2+} exchange mechanism although in experiments with SDS pretreatment a disturbance of membrane lipidic architecture may also by responsible for the diminution of Na^+—Ca^{2+} exchange rates.

Graphical analysis of the pronase stimulated transport according to Lineweaver-Burk's plots revealed that pronase did not change the K_m value for Ca^{2+} but it increased V_{max} only (Fig. 4).

Discussion

In rat brain microsomal vesicles, Na^+—Ca^{2+} exchange could be stimulated by their partial digestion with pronase similarly as in myocardial sarcolemma (Phillipson and Nishimoto 1982); there are only quantitative differences between both structures. The stimulation of Na^+—Ca^{2+} exchange has been ascribed to the proteolytic action of proteinases which should modify the Na^+—Ca^{2+} exchange system by increasing its affinity to calcium as well as by increasing V_{max} (Phillipson and Nishimoto 1982). The above authors reached maximal stimulation of calcium

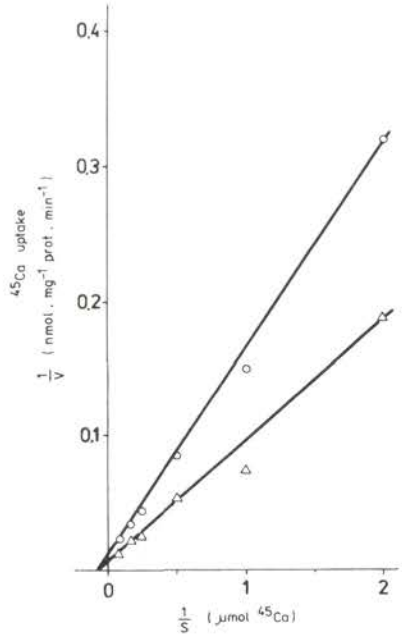

Fig. 4. Lineweaver-Burk's plots of reciprocal values of velocities ($1/V$) against Ca^{2+} concentrations ($1/S$) in rat brain microsomal membranes pretreated with pronase in the ratio $1:10^3$ (△—△) and in control membranes (○—○). Mean values of 3 independent experiments.

transport by splitting off 5—9.7 % of the membrane proteins. In our experiments a significant increase in calcium exchange occurred when about 11 % of the membrane proteins were liberated by pronase pretreatment (Fig. 2). An excess of Trasylol in the presence of pronase resulted in a diminished effect of the latter on the Na^+—Ca^{2+} exchange system; since Trasylol prevents proteolysis it can be assumed that some membrane proteins were involved in the Na^+—Ca^{2+} counter-transport. This system, like the sodium channel (Barnola and Forres 1983), is relatively resistant to digestion with proteolytic enzymes. It is probable that following mild proteolysis with pronase some Na^+—Ca^{2+} exchangers present in the membranes become accessible to the substrate and thus an increased $Na^+ - Ca^{2+}$ exchange rate occurred. There also is another possibility: since pronase splits off inactive polypeptides of high molecular weight, active proteins of the exchanger prevail in the membranes and therefore a higher Na^+—Ca^{2+} exchange rate is observed.

Our results indicate that proteins with molecular weights between 45,000 and 20,000 are involved in the Na^+—Ca^{2+} exchange system in brain microsomal membranes (Fig. 2 and 3). Similar conclusions could de drawn by Wakabayashi

and Goshima (1982) who inserted solubilized sarcolemmal proteins into arteficial lipidic vesicles; they found 5—6 protein bands with molecular weights between 20,000 and 40,000 in the active vesicles.

Bilateral ligature of both common carotid arteries for 2—4 h, EEG depression elicited in the ischaemic brain cortex at manifold prolonged negative phase of wave depression (Ruščák and Ruščáková 1971), or complete brain ischaemia had no influence on the sodium dependent calcium transport. Under these conditions an increased intracellular calcium content (Meldrum 1983) should stimulate the activities of endogenous proteinases (Siesjö 1983) and, through their activation, a stimulation of calcium transport into the vesicles might be expected like in in vitro experiments with pronase digestion. Since this was not the case, we assumed that the pronase stimulation of the calcium transport only occurred in vesicles with a right side out orientation with the superficial proteins being partially digested with pronase; these proteins are not accessible to proteinases activated by calcium present inside the cells.

Phillipson and Nishimoto (1982) have assumed that proteinase pretreatment of sarcolemmal membranes would increase, first of all, the affinity of calcium to the carrier, and much less V_{max}. Contrary to this, our results have indicated that there was no change in the K_m value for calcium in the membranes pretreated with pronase, but only an increase in V_{max} (Fig. 4). A probable explanation of this phenomenon could be looked for in the demasking of preexisting carrier units in the membranes after the liberation of high molecular weight proteins from the membranes or in a relative increase of carrier units due to the liberation of inactive polypeptides from the membranes.

References

Barnola F. V., Forres M. E. (1983): Effect of proteolysis on the activity of the sodium channel in isolated lobster nerve membrane. J. Neurosci. Res. **10,** 73—81

Laemli M. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227,** 680—685

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265—275

Luciani S. (1984): Reconstruction of the sodium-calcium exchanger from cardiac sarcolemmal vesicles. Biochim. Biophys. Acta **772,** 127—134

Meldrum B. (1983): Selectively vulnerable neurons and mitochondrial calcium accumulation in status epilepticus. J. Neurochem. **41** (suppl.), S 71

Phillipson K. D., Nishimoto N. Y. (1982): Stimulation of Na^+—Ca^{2+} exchange in cardiac sarcolemmal vesicles by proteinase pretreatment. Am. J. Physiol. **243,** C191—C195

Ruščák M., Ruščáková D. (1971): Metabolism of the Nerve Tissue in Relation to Ion Movements in Vitro and in Situ. Publ. House Slovak Acad. Sci., Bratislava

Schellenberg G. D., Swanson P. D. (1981): Sodium dependent and calcium dependent calcium transport by rat brain microsomes. Biochim. Biophys. Acta **648,** 13—27

Schellenberg G. D., Swanson P. D. (1982): Solubilization and reconstitution of membranes containing the Na^+—Ca^{2+} exchange carrier from rat brain. Biochim. Biophys. Acta **690,** 133—144

Siesjö B. K. (1983): Neurochemical effects of hypoxia and hypoglycaemia. J. Neurochem. **41** (suppl.), S 143

Wakabayashi S., Goshima K. (1982): Partial purification of Na^+—Ca^{2+} antiporter from plasma membrane of chick heart. Biochim. Biophys. Acta **693,** 125—133

Received July 18, 1984/Accepted November 20, 1984