Inhibition of Na^+—Ca^{2+} Exchange by Calcium Antagonists in Rat Brain Microsomal Membranes

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Abstract. Na^+—Ca^{2+} exchange rates were studied in native and/or pronase pretreated rat brain microsomal membranes in the presence of calcium channel antagonists verapamil, nimodipine and nifedipine. In native membranes all the substances used inhibited Na^+—Ca^{2+} exchange. A relatively stronger inhibition was observed in membranes pretreated with pronase. The values of K_i for nimodipine and nifedipine did not change and it fell to about one half for verapamil. Lineweaver-Burk's plots have revealed that the verapamil inhibition in native membranes as well as in pronase pretreated ones was of a non-competitive type; K_m for calcium oscillated around 15 μmol . l^{-1}. It is suggested that the inhibition strength depends on the access of inhibitors to the membrane binding sites as well as on the solubility of inhibitors in membrane lipids.

Key words: Na^+—Ca^{2+} exchange — Calcium antagonists — Verapamil — Nimodipine — Nifedipine — Pronase digestion — Rat brain microsomal membranes

Introduction

Na^+—Ca^{2+} countertransport in cytoplasmic membranes involves a complex system of polypeptides and lipids (Phillipson and Nishimoto 1982; Phillipson at al. 1983). The Na^+—Ca^{2+} exchanger, though probably chemically differing from the calcium channel (Barzilai et al. 1984; Curtis and Catterall 1984; Hale et al. 1984), has many common features with the channel: it can be inhibited by Me^{2+} ions, La^{3+} (Coutinho et al. 1984; Nachshen and Blaustein 1980; Orlický et al. 1985) and some calcium channel antagonists (Erdreich et al. 1983; Liron et al. 1985; Ueda 1983); on the other hand, it can be stimulated by partial digestion of the membranes with proteolytic enzymes (Phillipson and Nishimoto 1982; Ruščák et al. 1985) or phospholipases (Phillipson et al. 1983). In experiments presented herein Na^+—Ca^{2+} exchange was studied in the presence of calcium antagonists verapamil and dihydropyridine derivatives nimodipine and nifedipine in native rat brain microsomal membranes and/or in membranes partially digested with pronase; this (after treatment) can significantly modify the properties of the Na^+—Ca^{2+}

exchanger (Phillipson and Nishimoto 1982; Ruščák et al. 1985). The experiments were aimed at testing the concentration dependences of both calcium antagonists and substrate on Na^+—Ca^{2+} exchange in both groups of the membranes.

Materials and Methods

Chemicals and materials used: sucrose, TEMED (Merck); β-mercaptoethanol, SDS, acrylamide, N,N'methylene-bis-acrylamide, TRIS, bovine serum albumin, glycine, EDTA, EGTA, PAGE calibration kit (Serva); pronase (Koch-Light); $^{45}CaCl_2$ s.a. 111 GBq/g Ca (Institute of Isotopes of Hungarian Academy of Sciences); verapamil (Orion); nifedipine, nimodipine (Gift of the Drug Research Institute, Modra); all other reagents of analytical grade were purchased from Lachema Brno; SLT-41 scintillation coctail Spolana, millipore glass microfibre filters GF/C (Whatman).

The microsomal membrane fraction was prepared from the brain cortex of Wistar rats. The brains of decapitated animals were excised in a cooled room and homogenized in a Potter-Elvehjem glass homogenizer with a teflon pestle in a ratio of 1 g of tissue in 8 ml of homogenizing medium of the following final composition (mmol . l^{-1}): sucrose 320; EDTA 5; EGTA 10; β-mercaptoethanol 2; pH of the medium was adjusted to 7.4 with 2 mol . l^{-1} TRIS solution. The homogenates were centrifuged for 30 min at 12,000xg in a MSE 17 centrifuge. After decanting the supernatants, the sediments were rehomogenized in a ratio 1:3 in the same medium and centrifuged under the same conditions as above. The combined supernatants were centrifuged for 1 h at 53,000xg in a VAC 602 ultracentrifuge using a 8 × 35 ml swing out rotor. The sediments of microsomal membranes were stirred in 5 mmol . l^{-1} sodium phosphate buffer and centrifuged as above. The obtained sediments were again stirred in 20 mmol . l^{-1} TRIS-HCl buffer, pH 7.4, and divided into 2 portions. One part of the specimens was left in 20 mmol . l^{-1} TRIS-HCl buffer, for 15 min at 37 °C, the other one was digested with pronase in a pronase to membrane protein ratio of 1:100. The membrane suspensions were then cooled on ice and centrifuged for 1 h at 100,000 × g and the sediments and supernatants were subsequently assayed for proteins according to Lowry et al. (1951).

The sediments of microsomal membranes were preloaded overnight in 160 mmol . l^{-1} NaCl solution + 20 mmol . l^{-1} TRIS-HCl, pH 7.4, and then used for the determination of Na^+—Ca^{2+} exchange rates according to Schellenberg and Swanson (1981). Forty to fifty μg of vesicular proteins preloaded with NaCl were transferred into 1 ml of equimolar NaCl or KCl solution buffered with TRIS containing $^{45}CaCl_2$ in final initial concentrations as given in the legends to the Table and Figures. When calcium antagonists were used, the membranes were preincubated under protected sunlight with the former for 5 min and then Na^+—Ca^{2+} exchange was determined. Verapamil was added dissolved in water, nimodipine and nifedipine were dissolved in dimethylsulphoxide. The Ca^{2+} uptake was terminated after 15 s of incubation at room temperature by rapid filtration of specimens through GF/C glass fibre filters. Prior to the filtration, the filters were washed twice with 2 ml of cooled KCl or NaCl solution (160 mmol . l^{-1}). Dried filters were suspended in SLT-41 solution and the radioactivity was counted in a LKB Rackbeta scintillation counter. The results were expressed as differences in calcium uptake in NaCl medium (blanks) against KCl medium in nmol $^{45}Ca^{2+}$. mg^{-1} prot . min^{-1}.

Proteins in the membranes were determined following solubilization of proteins with 1 % SDS solution, bovine serum albumin being used as a standard.

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

Abbrevations used: EGTA — ethyleneglycol-bis(2 aminoaethylaether)-N,N'tetramethylenediamine; SDS — sodium dodecylsulphate; TEMED — N,N,N',N' tetramethylenediamine; TRIS — tri(hydroxymethyl) aminomethane; EDTA — ethylenediamine tetraacetic acid.

Results

The first series of experiments were designed to confirm our previous observations of a significant inhibition of Na^+—Ca^{2+} exchange in the presence of 1 mmol . l^{-1} verapamil (Orlický et al. 1985). In addition to verapamil, nimodipine and nifedipine at the same concentrations were also tested. Na^+—Ca^{2+} exchange was studied in native membranes and in membranes partially digested with a pronase protein ratio of 1 : 100. The results summarized in Table 1 have shown that, in native membranes, a significant decrease in Na^+—Ca^{2+} exchange could be observed with verapamil and nifedipine only, and not with nimodipine. If, however, the above antagonists were added to the membranes partially digested with pronase all the calcium antagonists used inhibited significantly and to a relatively larger extent the Na^+—Ca^{2+} exchange. The amount of proteins split off from the membranes with pronase after 15 min incubation reached about 20—22 % of the total membrane proteins. The above antagonists did not influence passive calcium binding to the membranes.

Table 1. Na^+—Ca^{2+} exchange rates in rat brain microsomal membranes in the presence of 1 mmol . l^{-1} calcium antagonists in native membranes (N) and in membranes partially digested with pronase (Pr). The results are expressed in terms of nmol . mg^{-1} prot . min^{-1}; arithmetical means ± S.E.M. of 5 different specimens are given. Initial $^{45}Ca^{2+}$ concentration 10 μmol . l^{-1}.

Ca^{2+} antagonists	0	Verapamil	Nimodipine	Nifedipine
N	10.1 ± 1.1	5.5 ± 0.8	8.5 ± 0.6	7.0 ± 0.7
Pr	9.6 ± 1	1.0 ± 0.1	4.0 ± 1.1	3.5 ± 0.5

In a further series of experiments, Na^+—Ca^{2+} exchange was studied in relation to the concentrations of the inhibitors in the medium. Half-maximal inhibition was observed with nimodipine and nifedipine at their concentrations lower than 2×10^{-4} mol . l^{-1} both in the native membranes and in the membranes partially digested with pronase; a relatively stronger inhibition was observed in the membranes pretreated with pronase at all the concentrations used (Fig. 1).

The effect of verapamil was then analysed in more detail with regard to its concentrations and to the concentrations of calcium in the media. Contrary to dihydropyridine derivatives, in the native membranes verapamil linearly inhibited Na^+—Ca^{2+} exchange in dependence on its concentrations in the medium with an I_{50} at about 5×10^{-4} mol . l^{-1} at different calcium concentrations. While verapamil at a concentration of 5×10^{-5} mol . l^{-1} had no effect on Na^+—Ca^{2+} exchange in the native membranes, this concentration significantly inhibited the exchange in the pronase pretreated membranes. A relatively stronger inhibition of Na^+—Ca^{2+}

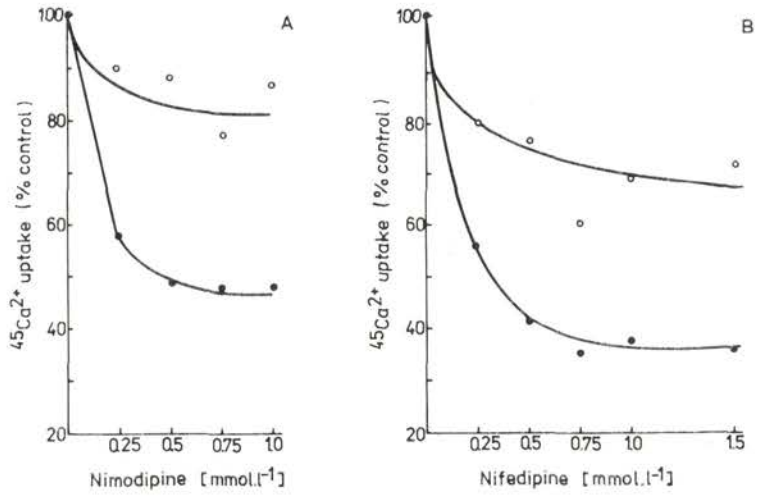

Fig. 1. Inhibition of Na^+—Ca^{2+} exchange rates in native (○—○) and pronase pretreated (●—●) rat brain microsomal membranes in the presence of different concentrations of nimodipine (*A*) or nifedipine (*B*). Mean values of 5 experiments related to the control value of 8.47 ± 1.4 mmol . mg^{-1} prot . min^{-1}. External $^{45}Ca^{2+}$ concentration 10 μmol . l^{-1}. Each point represents mean value of 5 independent measurements. The S.E.M. values were between 9—14 % of the means.

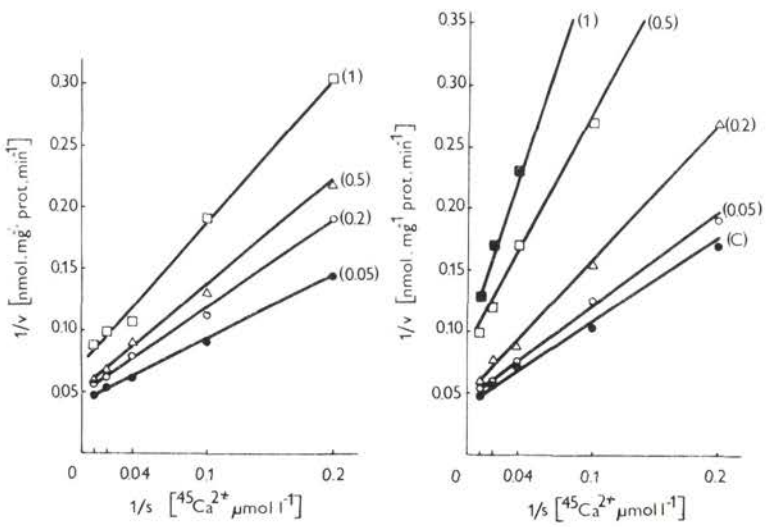

Fig. 2. Lineweaver-Burk's plots of reciprocal values of velocities ($1/V$) against substrate concentrations ($1/S$) at different external verapamil concentrations (indicated in parentheses in μmol . l^{-1}). *Left panel:* native membranes; *right panel:* membranes partially digested with pronase.

exchange was observed at all the verapamil concentrations used in the pronase pretreated membranes. In the pronase pretreated membranes, half-maximal inhibition was observed at about 2×10^{-4} mol . l^{-1} of verapamil. An about 50 % inhibition was observed with 1 mmol . l^{-1} verapamil in the native membranes, while in the membranes partially digested with pronase the same verapamil concentration, inhibited nearly completely the Na^+—Ca^{2+} countertransport. The Na^+—Ca^{2+} exchange rates compared at various contentrations of verapamil and calcium have shown that in both the native and partially digested membranes, verapamil is a non-competitive inhibitor with regard to calcium ; it did not change the K_m for calcium (which was within a concentration range 15—20 μmol . l^{-1}), but it diminished the V_{max} in dependence on its concentration in the external medium (Fig. 2).

Discussion

Verapamil is a blocker of slow sodium and calcium channels in cell membranes (Ferry and Glossman 1982; Kojima and Sperelakis 1983) and it also blocks Na^+—Ca^{2+} exchange. The inhibitory effect of verapamil on Na^+—Ca^{2+}exchange varies greatly in dependence on the origin of the membranes. In lymphocytic membranes half-maximal inhibition has been found at 5 mmol . l^{-1} verapamil (Ueda 1983), while a considerably lower concentration is needed to obtain the same level of inhibition in brain cell membranes. Erdreich et al. (1983) have reported a K_i for verapamil value of 175 μmol . l^{-1} in the synaptosomal membranes, and a complete inhibition of Na^+—Ca^{2+} exchange at 500 μmol . l^{-1} verapamil. A similar K_i for verapamil in brain microsomal membranes was found by Liron et al. (1985). In our experiments half-maximal inhibition of Na^+—Ca^{2+} exchange by verapamil in native membranes was observed at 500 μmol . l^{-1} and 200 μmol . l^{-1} in pronase pretreated membranes. In the native membranes, a nearly total blockade of Na^+—Ca^{2+} exchange by verapamil could never be obtained in contrast to the pronase digested membranes (Fig. 2). Verapamil was a significantly more potent inhibitor in both the native and the pronase pretreated membranes (Fig. 1 and 2) as compared with the dihydropyridine derivatives nimodipine and nifedipine.

The verapamil inhibition of Na^+—Ca^{2+} exchange in our experiments was of the non-competitive type with regard to calcium. This finding is contradictory to the results obtained by Liron et al. (1985) who found a competitive type of inhibition by verapamil in brain microsomal membranes. A non-competitive type of inhibition of Na^+—Ca^{2+} exchange by verapamil seems more probable as it is known that verapamil also inhibits membrane cell processes which are independent of calcium but dependent on sodium only (Erdreich et al. 1983; McGee and Schneider 1979). A non-competitive type of Ca^{2+} transport are also suggested by

data reported by Lamers (1985) who has shown that calcium binds to the membranes on sites different from those for calcium antagonists. A negative allosteric effect of verapamil and, maybe, of other calcium antagonists on the Na^+—Ca^{2+} exchange system in brain microsomal membranes can therefore be suggested. A non-competitive inhibition of Na^+—Ca^{2+} exchange is also suggested by the results shown in Fig. 2; it is clear from this Figure that verapamil did not change the K_m value for calcium but inhibited V_{max} in both the native microsomal and the pronase pretreated membranes. The K_m value for calcium was in the range of 15—20 μmol . l^{-1} in both types of the membranes; this value is very close to or identical with that for the Na^+—Ca^{2+} exchanger in the nerve tissue (Michaelis and Michaelis 1983; Schellenberg and Swanson 1981; Orlický et al. 1985).

A relatively stronger inhibition of Na^+—Ca^{2+} exchange was observed in the membranes pretreated with pronase in the presence of calcium antagonists used. Following pronase pretreatment, the membranes lose the ability of ATP-driven Ca^{2+} accumulation, Na^+—K^+-ATPase activity decreases (unpublished results) and large polypeptide molecules are split off (Ruščák et al. 1985). A stronger inhibition by verapamil as compared with nimodipine and nifedipine can be due to the fact that verapamil is more soluble in the lipids (Pang and Sperelakis 1983); a higher effectiveness of calcium antagonists in pronase pretreated membranes may be due to the degradation of membrane proteins of large molecular weight with pronase (Ruščák et al. 1985). The exchanger protein and/or the membrane lipids thus become more easily accessible for calcium antagonists. Antagonists embedded to a greater extent in lipids of partially digested membranes can change the membrane fluidity and reduce in this way the activity of the Na^+—Ca^{2+} exchanger.

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