Effects of Sulfhydryl Reagents on Na^+—Ca^{2+} Exchange in Rat Brain Microsomal Membranes

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Abstract. Effects of six thiol reagents with different physico-chemical properties were tested on the Na^+-dependent $^{45}Ca^{2+}$ transport into the rat brain microsomal membrane vesicles. The mercurials *p*-chlormercuribenzoate and Mersalyl effectively inhibited $^{45}Ca^{2+}$ uptake with IC_{50} values in the order of 10^{-4} mol.l^{-1} in the medium. N-ethylmaleimide and its more lipophilic analog N-(4-(2-benzoxazolyl)phenyl)maleimide were much less effective at the same concentrations. 2,2′-dithiodipyridine markedly reduced $^{45}Ca^{2+}$ uptake already at concentrations below 10^{-4} mol.l^{-1}, whereas 5,5′-dithiobis-2-nitrobenzoate in a concentration range $10^{-6} - 10^{-3}$ mol.l^{-1} was a weak inhibitor. Inhibitory effects of the most potent inhibitors *p*-chlormercuribenzoate and 2,2′-dithiodipyridine were readily reversed by 1 mmol.l^{-1} dithiothreitol. The results suggest that free SH groups of membrane polypeptides are involved in the functioning of the Na^+—Ca^{2+} exchanger in the nerve tissue cell membranes.

Key words: Sulfhydryl reagents — Na^+—Ca^{2+} exchange — Microsomal rat brain membranes

Introduction

The Na^+—Ca^{2+} exchange mechanism plays a key role in the control of intracellular Ca^{2+} homeostasis and/or Ca^{2+} movements across the membranes of excitable as well as nonexcitable tissues (Baritt 1981; Åkerman 1982; DiPolo and Beaugé 1983; Ueda 1983; Blaustein 1984; Carafoli 1984; Kaczorowski et al. 1984). The molecular mechanisms of Na^+—Ca^{2+} exchange have not yet been fully understood. Many authors have suggested that membrane proteins are involved in the Na^+—Ca^{2+} countertransport system (Phillipson and Nishimoto 1982; Luciani 1984; Ruščák et al. 1985). Blockade of the SH groups in membrane polypeptides modifies the transport and excitation processes (Marquis and Mautner 1974; Strager 1977; Baumgold et al. 1978): reagents reacting with sulfhydryl groups were found to affect Ca^{2+}-pump and channel functions (Yasuoka-Yabe and Kawakita 1983; Goll et al. 1984; Brandt et al. 1985); no direct evidence has been published, however, for the participation of sulfhydryl groups

in the Na^+—Ca^{2+} exchange system. In our present study, several reagents with different binding properties for sulfhydryl groups were tested on the Na^+-dependent $^{45}Ca^{2+}$ uptake in rat brain microsomal membrane vesicles. Our results indicated that thiol groups are also involved in Na^+—Ca^{2+} exchange functions. A preliminary report of these findings has been published in an abstract form (Orlický et al. 1985).

Materials and Methods

Chemicals

Sucrose, 1,4-dithiothreitol (Merck), 5,5'-dithiobis-(2-nitrobenzoic acid), EGTA, N-ethylmaleimide, N-(4-(2-benzoxazolyl)phenyl)maleimide (Serva), 2,2'-dithiodipyridine (Fluka), Mersalyl (Sigma), 2-Mercaptoethanol (Koch-Light), scintillation cocktail SLT-41 (Spolana Neratovice), $^{45}CaCl_2$ (specific radioactivity 111 GBq/g Ca) (Radioisotope Laboratory, Hungarian Academy of Sciences). Other chemicals used were of analytical grade (Lachema Brno).

Methods

Microsomal membranes were isolated from the cortex and midbrain of Wistar rats weighing 150—200 g, as reported previously (Ruščák et al. 1985). The microsomal membranes were suspended in 160 mmol.l^{-1} NaCl, 20 mmol.l^{-1} Tris-HCl, pH 7.4, and left overnight at 0—2 °C for passive Na^+ loading at about 2 mg protein/ml.

Measurements of ^{45}Ca uptake

Membrane vesicles, loaded with Na^+ were preincubated at 25 °C for 5 min and subsequently thiol reagents were added in concentrations given in legends to figures. Reagents requiring an organic solvent were dissolved in dimethylsulphoxide to give a final concentration of 0.1 %. The samples were than incubated for additional 5 min. Twenty μl (40—50 μg proteins) of the membrane vesicles were diluted 50-fold into 1 ml of 160 mmol.l^{-1} NaCl, 20 mmol.l^{-1} Tris-HCl, pH 7.4, 10 μmol.l^{-1} $^{45}CaCl_2$ or 160 mmol.l^{-1} KCl, 20 mmol.l^{-1} Tris-HCl, pH 7.4, 10 μmol.l^{-1} $^{45}CaCl_2$. After 15 s the uptake of $^{45}Ca^{2+}$ was terminated by rapid filtration through Whatman GF/C glass fibre filters. The filters were washed twice with 4 ml of ice-cold 160 mmol.l^{-1} KCl, 20 mmol.l^{-1} Tris-HCl, pH 7.4, and dried in hot air. SLT-41 was added and the radioactivity was determined by liquid scintillation counting in a LKB Rackbeta equipment.

Reversal of the effects of p-chlormercuribenzoate and 2,2'-dithiodipyridine

Microsomal membrane vesicles were incubated with PCMB or DTDP at 25 °C for 5 min; subsequently dithiothreitol (final concentration 1 mmol.l^{-1}) was added, and the incubation was continued for additional 5 min. ^{45}Ca uptake was measured as described above.

The protein content was estimated by the method of Lowry et al. (1951) in the presence of 0.5 %

Abbrevations used: NEM, N-ethylmaleimide; PCMB, para-chlormercuribenzoate; NPBM, N-(4-(2-benzoxazolyl)phenyl)maleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTDP, 2,2'-dithiodipyridine; EGTA, ethylenglycol bis(-aminoethyl ether)-N,N'-tetraacetic acid; DTT, 1,4-dithiothreitol.

sodium dodecylsulphate, with bovine serum albumine used as a standard. Results were expressed in terms of differences in calcium uptake in NaCl against KCl media in nmol ^{45}Ca per mg protein per sec.

All results were statisticaly treated using Student's *t*-test.

Since relatively large differences (0.25—0.45 nmol . $mg^{-1} . s^{-1}$) in Na^+—Ca^{2+} exchange rates were found between membranes prepared on various days with the standard procedure, (also see Barzilai et al. 1984), specimens prepared on the same day from different rat brains were always compared with each other. The summarized results are shown in figures.

Results

Fig. 1 shows the chemical structures of the SH reagents tested.

p-chlormercuribenzoate (PCMB) (Fig. 2*A*) was very effective in inhibiting $^{45}Ca^{2+}$ uptake into microsomal membrane vesicles with an IC_{50} value of about 100 μmol . l^{-1}, i.e. 100 nmol of PCMB were required to obtain half-maximal inhibition per mg of microsomal membrane proteins. Another mercurial thiol reagent Mersalyl (Fig. 2*A*) was less effective than PCMB. The amount of Mersalyl required to elicit half-maximal inhibition was about 600 μmol . l^{-1}

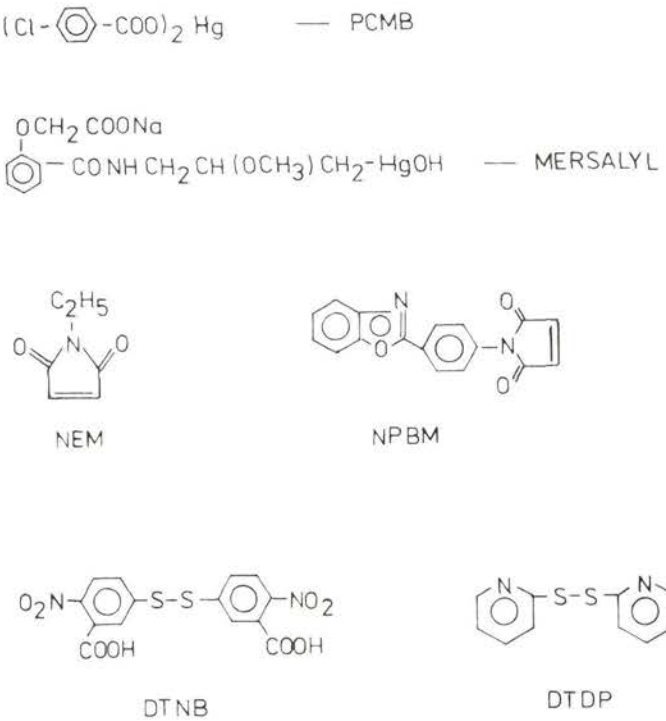

Fig. 1. Chemical structure of thiol reagents: PCMB, *p*-chlormercuribenzoate; Mersalyl; NEM, N-ethylmaleimide; NPBM, N-(4-(2-benzoxazolyl)phenyl)maleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTDP, 2,2′dithiodipyridine.

(600 nmol/mg protein). The irreversibly reacting SH-reagent, N-ethylmaleimide (NEM) (Fig. 2*B*) was less effective. At the maximal concentration used (10 mmol.l^{-1} NEM), it decreased $^{45}Ca^{2+}$ uptake by about 30—40 % only. Titration of Na^+-dependent $^{45}Ca^{2+}$ uptake with N-(4-(2-benzoxazolyl)phenyl)-maleimide, the most lipophilic analog of NEM, resulted in a typical inhibition curve (Fig. 2*B*), showing an initial lag-phase, followed by a slight inhibition. 5,5′-dithiobis-2-nitrobenzoate (DTNB) at the maximal concentration used (1 mmol.l^{-1}) decreased ^{45}Ca uptake by about 10—15 % (Fig. 2*C*). Another

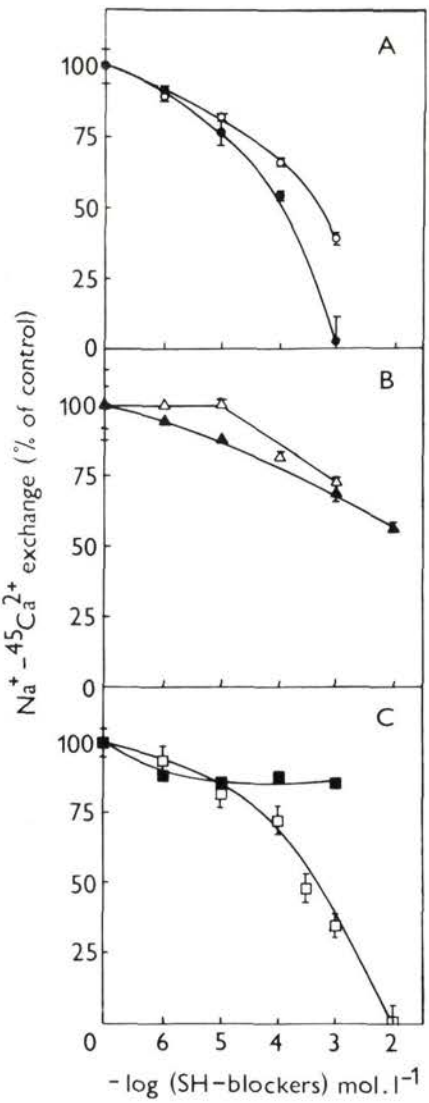

Fig. 2. The effects of various SH-blockers on Na^+-dependent $^{45}Ca^{2+}$ uptake into rat brain microsomal membrane vesicles. Each point represents mean ± S.E.M. from 4—6 experiments in terms of percentage of control values (100 %). The mean control values were for PCMB (●—●) and Mersalyl (○—○) (Fig. 1*A*) 0.35 ± 0.05; for NEM (▲—▲) and NPBM (△—△) (Fig. 1*B*) 0.30 ± 0.05 and for DTNB (■—■), DTDP (□ □) (Fig. 1*C*) 0.35 ± 0.04 nmol.$mg^{-1}.s^{-1}$.

reagent able to modify sulfhydryl groups, 2,2′-dithiodipyridine (DTDP) markedly inhibited $^{45}Ca^{2+}$ uptake only at much higher concentrations (0.1—1 mmol.l^{-1}) with IC_{50} of about 0.5 mmol.l^{-1} (0.5 μmol/mg protein) (Fig. 2*C*).

Reversibility of the effects of PCMB and DTDP

The reversibility of the inhibition of ^{45}Ca uptake into microsomal membrane vesicles from rat brain by PCMB and DTDP was studied using 1,4-dithioth-

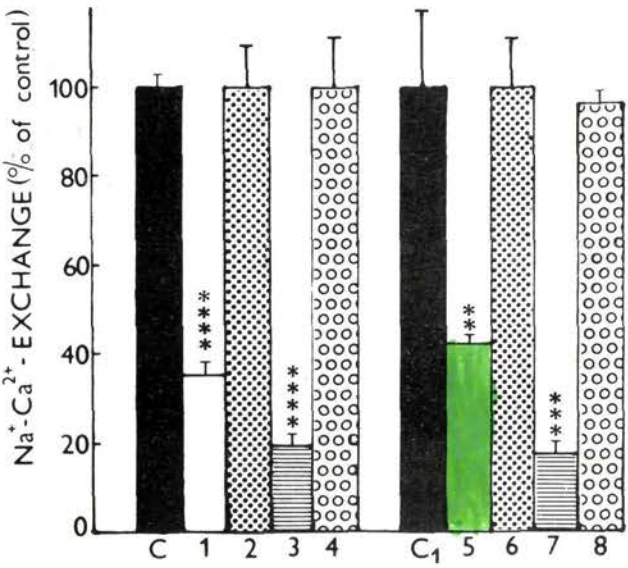

Fig. 3. The reversal effects of SH-blockers inhibition of Na^+-dependent $^{45}Ca^{2+}$ uptake in rat brain microsomal membrane vesicles by 1,4-dithiothreitol (DTT). C: control value in the absence of PCMB was 0.35 ± 0.05 nmol.mg^{-1}.s^{-1}; 1: in the presence of 0.1 mmol.l^{-1} PCMB; 2: 0.1 mmol.l^{-1} PCMB 1 mmol.l^{-1} DTT; 3; 0,5 mmol.l^{-1} PCMB; 4: 0.5 mmol.l^{-1} PCMB, 1 mmol.l^{-1} DTT; C_1 — control value in the absence of DTDP was 0.030 ± 0.04 . nmol . mg^{-1} . s^{-1}; 5: 0.5 mmol.l^{-1} DTDP; 6: 0.5 mmol.l^{-1}, 1 mmol.l^{-1} DTT; 7: 1 mmol.l^{-1} DTDP; 8: 1 mmol.l^{-1} DTDP, 1 mmol.l^{-1} DTT. $^{**}P < 0.05$, $^{***}P < 0.02$, $^{****}P < 0.01$.

reitol (1 mmol.l^{-1}). Fig. 3 shows that pretreatment of microsomal membrane vesicles with 0.1 or 0.5 mmol.l^{-1} PCMB resulted in the inhibition of ^{45}Ca uptake; the addition of DTT, however, resulted in restoration of ^{45}Ca influx to control values. Similar results were obtained with 0.5 and 1 mmol.l^{-1} DTDP, respectively. Dithiothreitol itself (1 mmol.l^{-1}) had no effect on $^{45}Ca^{2+}$ uptake into microsomal membrane vesicles.

Discussion

Reagents modifying the SH side groups in polypeptides have been used for the studies of structure- and function relationships of many membrane enzymes

(Means and Fedney 1971; De Grip and Daemen 1982). It was suggested that a limited number of sulfhydryl groups of the sarcoplasmic reticulum Ca-pump could be selectively modified by titration with thiol reagents. Further detailed studies of SR membrane vesicle ATPase with site-specific labeling of SH groups by fluorescence and paramagnetic probes have shown that distinct sulfhydryl groups are involved in the Ca-dependent conformational changes at the transport site (Anderson et al. 1980; Yasuoka-Yabe and Kawakita 1983). The free sulfhydryl groups are also involved in maintaining the tertiary structure of the Ca^{2+} channel in skeletal muscle (Goll et al. 1984). In the present studies, various thiol reagents were used in examining the Na^+—Ca^{2+} exchange rates. These compounds were able to reduce the Na^+—Ca^{2+} countertransport, which was to a certain extent dependent on their solubilities in water or organic solvents. Reversibly reacting water soluble mercurials like PCMB and Mersalyl were the most potent inhibitors of Na^+—Ca^{2+} exchange in rat brain microsomal membrane vesicles. The ability of water soluble mercurials PCMB and Mersalyl, which are only partially membrane-permeable (Wehrle and Pedersen 1981), to block ^{45}Ca uptake seems to indicate that the reactive SH groups of the carrier protein are oriented towards the water phase of the plasma membrane. The irreversibly reacting lipophilic reagents were effective in reducing ^{45}Ca uptake at much higher concentrations only. These compounds react most favourably with sulfhydryl groups in hydrophobic chains but they undergo a side reaction with amino groups as well (Brewer and Riehm 1967; Rack et al. 1984).

The IC_{50} values for the effective SH group reagents ranged between 100—500 $\mu mol \cdot l^{-1}$, corresponding to 0.1—0.5 $nmol/\mu g$ protein. Similar results were obtained by Höther and Kadembach (1984) for pig heart mitochondrial phosphate carrier, and by Troeger et al. (1984) for the GABA transporter in rat brain synaptosomes and for the high affinity binding of (^3H)Verapamil with *p*-chlormercuri-phenylsulphonic acid and *N*-ethylmaleimide at the drug receptor sites within the Ca^{2+} channel in skeletal muscle microsomes (Goll et al. 1984). Low concentrations of *p*-chlormercuribenzoate, but not of N-ethylmaleimide, were able to block the binding of the agonist and antagonist of central α_1 and α_2 adrenoreceptors in rat brain membranes (Quennedey et al. 1984). It is, however, worth mentioning that higher concentrations of SH reagents may effect other membrane functions: they can reduce the transmembrane electrical potential or react with any accessible SH group and thus modify many related functions (Troeger et al. 1984; Rack et al. 1984).

Based on the known reactivity of the mercurial and DTDP reagents (Grasceti and Murray 1967), their inhibitory effects on Na^+—$^{45}Ca^{2+}$ exchange could be explained by their direct interaction with sulfhydryl groups of the carrier, or

indirectly by a total blockade of membrane protein SH groups, which result in reversible inactivation of the carrier. This conclusions has also been supported by the finding (Fig. 3) that the inhibition was reversed in the presence of the reducing agent dithiothreitol. The effect of NEM and NPBM may also have been due to reactions with specific amino groups of the carrier or other related membrane protein constituents. Another possibility would be that the inhibition was caused by non-covalent interactions of hydrophobic ring structures of these reagents with further unspecified membrane structures (Rack et al. 1984).

In summary, our results have shown that interactions of sulfhydryl reagents with membrane proteins result in functional inactivation of the Na^+—Ca^{2+} exchanger. Our results are insufficient to point to the exact localization of the site involved. Further studies on the location of the reactive SH groups in the solubilized, purified and reconstituted Na^+—Ca^{2+} exchanger would provide a solid basis for interpreting these results.

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