Journal of Bioenergetics and Biomembranes, Vol. 22, No. 5, 1990

Effects of Plasma Membrane Oxidoreductases on Ca^{2+} Mobilization and Protein Phosphorylation in Rat Brain Synaptosomes

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Received November 1, 1989; revised February 7, 1990

Abstract

We have investigated the possible role of plasma membrane oxidoreductases in the Ca^{2+} export mechanisms in rat brain synaptic membranes. Ca^{2+} efflux in nerve terminals is controlled both by a high-affinity/low capacity Mg-dependent ATP-stimulated Ca^{2+} pump and by a low affinity/high capacity ATP-independent Na^+–Ca^{2+} exchanger. Both Ca^{2+} efflux mechanisms were strongly inhibited by pyridine nucleotides, in the order NADP > NAD > NADPH > NADH with IC_{50} values of ca. 10 mM for NADP and ca. 3 mM for the other agents in the case of the ATP-driven Ca^{2+} pump and with IC_{50} values between 8 and 10 mM for the Na^+–Ca^{2+} exchanger. Oxidizing agents such as DCIP[3] and ferricyanide inhibited the ATP-driven Ca^{2+} efflux mechanism but not the Na^+–Ca^{2+} exchanger. In addition, full activation of plasma membrane oxidoreductases requires both an acceptor and an electron donor; therefore the combined effects of both substrates added together were also studied. When plasma membrane oxidoreductases of the synaptic plasma membrane were activated in the presence of both NADH (or NADPH) and DCIP or ferricyanide, the inhibition of the ATP-driven Ca^{2+} pump was optimal; by contrast, the pyridine nucleotide-mediated inhibition of the Na^+–Ca^{2+} exchanger was partially released when both substrates of the plasma membrane oxidoreductases were present together. Furthermore, the activation of plasma membrane oxidoreductases also strongly inhibited intracellular protein phosphorylation in intact synaptosomes, mediated by either *c*AMP-dependent protein kinase, Ca^{2+} calmodulin-dependent protein kinases, or protein kinase C.

Key Words: Plasma membrane oxidoreductases; neurotransmitters; cellular signaling; calcium; protein phosphorylation.

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[3]Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylenglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DCIP, dichlorophenol-indophenol.

0145-479X/90/1000-0645$06.00/0

Introduction

Excitability in neurons involves the stimulation of cation flux by neurotransmitters or neuroactive agents to bring about a change in the membrane electrical potential via an electrogenic process. The conductivity change reflects permeability changes mobilizing the steady-state gradients of ions such as H^+, Na^+, K^+, and Ca^{2+}. The mobilization of Ca^{2+} in synaptic plasma membrane is known to be mediated by three mechanisms (DiPolo and Beaugé, 1988): (a) a high-affinity/low-capacity (ATP + Mg)-dependent, vanadate-sensitive Ca^{2+} transport process, which is a Ca^{2+}-extruding plasma membrane (Ca^{2+} + Mg^{2+})-ATPase, analogous to those found in other plasma membranes: (b) a fully reversible Na^+–Ca^{2+} exchange mechanism, also believed to function in excitable membranes as an important Ca^{2+} extrusion mechanism with low affinity but high capacity; (c) a membrane potential-sensitive Ca^{2+} flux or voltage-dependent Ca^{2+} channel, which functions in the intact terminal to permit Ca^{2+} entry in response to depolarization. These two membrane transport systems are independent mechanisms used to pump Ca^{2+} out of the cell. Studies have shown that both mechanisms, which function in a single population of vesicles, derive from the plasma membrane and are operative in Ca^{2+} efflux.

On the other hand, pyridine nucleotides, serving as electron carriers in oxidation–reduction reactions, are vital in the regulation of the redox state of the cell. Many cellular functions, including mobilization of celluar Ca^{2+}, depend critically on the proper maintenance of the redox state. For example, it has been shown that glucagon can mobilize Ca^{2+} from perfused liver only if the redox state is within a specific range (Rasheed and Patel, 1987). On the other hand, the mobilization of intracellular Ca^{2+} can in turn modulate the redox state of the cell (Epel *et al.*, 1981; Poenie *et al.*, 1985; Eisen *et al.*, 1984), producing rapid phosphorylation of the cellular NAD^+ to $NADP^+$, part of which is subsequently reduced to NADPH through the pentose phosphate shunt (Epel, 1964). The overall effect is that the redox state of the cell becomes more reduced, which has been implicated to be important in the initiation and maintenance of cellular DNA synthesis (Whitaker and Steinhart, 1981).

In this paper we present evidence for a further mechanism of action of pyridine nucleotides by means of regulation of the transport systems responsible for Ca^{2+} extrusion through the plasma membrane. Plasma membranes are endowed with transplasma-membrane redox systems (Crane *et al.*, 1987, 1989), which are related to several vital functions, including control of cell growth (Sun *et al.* 1985), facilitation of iron uptake (Bienfait, 1985), defense against bacteria (Segal *et al.* 1983), but also to post-receptor signal transduction and membrane (de-)polarization, promoting ion or

amino acid transport or proton transfer across plasma membranes (Loew and Werner, 1976). Several NADH-dehydrogenases are present in the same population of synaptic membrane vesicles, and their specific function is still unknown (Treichler and Dreyer, 1987). This series of studies was undertaken to determine whether synaptic NADH-dehydrogenases affect in some manner the Ca^{2+} mobilization in synaptic nerve terminals. This paper shows that plasma membrane oxidoreductases from excitable membranes, besides regulating diverse transport mechanisms for Ca^{2+} mobilization, also inhibit intracellular protein phosphorylation.

Material and Methods

Materials

Radiochemicals were from Radiochemical Centre Amersham. All other reagents were from Sigma or Boehringer.

Preparation of Synaptic Membranes

Adult Sprague-Dawley rats (180–220 g) were used throughout this study. The rats were decapitated and the whole brains were soaked in 10 volumes ice-cold 0.32 M sucrose solution. Synaptic membranes were prepared as described by Cotman and Matthews (1971a, b) modified by Lopez-Perez *et al.* (1981). The brains were homogenized at 20% w/v in 0.32 M sucrose and diluted to 7% (w/v) for centrifugation at 1100 × *g* for 5 min. The supernatant was centrifuged at 17,000 × *g* for 10 min, the crude mitochondrial fraction was suspended in 10% sucrose, and pure synaptosomes were prepared by phase separation according to Lopez-Perez *et al.* (1981). A 5.4-g portion of this suspension was mixed with 32.0 g of poly(ethylene glycol)-Dextran T500, and centrifuged for 5 min at 1000 × *g*. The upper layer, enriched in synaptosomes, was overlayed on an equal volume of previously prepared lower phase and recentrifuged for 5 min at 1000 × g. The synaptosomes were sedimented by centrifugation for 20 min at 40,000 × *g* and the pellet resuspended in 0.32 M sucrose at ca. 60–80 mg protein per ml and frozen dropwise into liquid nitrogen until use.

Ca^{2+} Transport Assays

The method of Michaelis *et al.* (1983) was used with minor modifications. Frozen synaptic membranes were thawed rapidly at 37°C for 10 min in the presence of 100 mM KCl and 5 mM Tris/HCl, pH 7.4. Aliquots (100–200 μg of protein) were transferred to the incubation medium containing 100 mM KCl, 5 mM Tris/HCl, 25 μg of oligomycin, and 0.1 mM ouabain

in a final volume of 1 ml. In addition the incubation buffer usually contained 100 μM $MgCl_2$, 100 μM ATP, and 1 μM isotopically diluted $^{45}CaCl_2$ unless otherwise indicated. Free Mg^{2+}, ATP, and Ca^{2+} concentrations were maintained in the micromolar or submicromolar range by means of the divalent cation-chelating buffer *trans*-cyclohexane-1,2-diamine-*N*,*N*,*N'*,*N'*-tetraacetic acid (CDTA) present at 0.2 mM concentration in all samples. The constants used in determining the amount of $MgCl_2$ and $CaCl_2$ to be added in the presence of CDTA and various ATP concentrations were according to Michaelis *et al.* (1987).

Samples were preincubated for 4 min at 38°C with all components of the assay system except for ATP. Transport activity was initiated by addition of ATP and allowed to proceed for up to 20 min. The activity was terminated by addition of 3 ml of an ice-cold solution containing 150 mM KCl, 10 mM Tris/KCl, and 1 mM $LaCl_3$ to inhibit Ca^{2+} efflux and filtration through cellulose nitrate filters (0.45 μm pore size). Filters were washed twice with 3 ml of the stop solution and the trapped Ca^{2+} determined by liquid scintillation spectometry. Samples incubated for the entire time in the absence of ATP were used to determine the background binding or flux, and the activity in these samples was substracted from that in the comparable ATP-containing samples for calculating the ATP-dependent Ca^{2+} transport.

Where the effects of the synaptic NADH-dehydrogenases on the ATP-dependent Ca^{2+} transport were examined, the incubation medium contained the appropriate substrates (pyridine nucleotides and/or ferricyanide or DCIP) at the concentrations indicated in the figures.

Ca^{2+} Influx and Efflux Experiments

The method of Gill *et al.* (1981) was used with the following modifications. All media present during pre-equilibration, Ca^{2+} influx, and Ca^{2+} efflux contained 1 mM $MgSO_4$, 5 mM Hepes buffered to pH 7.4 with Tris (approximately 2.7 mM), and either 100 mM NaCl, KCl, or 100 mM sucrose, unless stated otherwise.

For Ca^{2+} influx experiments, aliquots of membrane vesicles (100 μl) were thawed at room temperature, diluted with 1.0 ml of appropriate medium, and allowed to pre-equilibrate for 10 min at 37°C. Vesicles were then centrifuged (5 min at 20,000 $\times$ *g*), rewashed with 1.0 ml of equilibration medium, and finally resuspended with 200 to 400 μl of the same medium. Ca^{2+} influx was initiated by adding 5 μm of pre-equilibrated vesicles to 100 μl of appropriate external medium containing the additions as described in the figures, together with 0.5 μCi of $^{45}CaCl_2$ (approximately 500,000 cpm) and 10 μM unlabeled $CaCl_2$ (unless otherwise stated) to give a specific activity for ^{45}Ca of 0.5 Ci/mmol. Influx continued at room temperature for 4 min or the time

specified and was terminated by dilution with 2.5 ml of ice-cold 0.15 M KCl and rapid filtration through cellulose acetate membrane filters (0.5 μm pore size). After further washing with 2.5 ml of KCl, filters were dissolved in scintillation fluid and counted.

The effects of synaptic NADH-dehyrogenases were tested by adding the appropriate substrates at the concentrations indicated into the incubation medium. Proper control devoid of substrates was always run in parallel within the same experiment.

Results are expressed as the amount of Ca^{2+} accumulated by vesicles with nonspecific adsorption to filters substracted.

Protein Phosphorylation

The procedure of Palfrey and Mobley (1987) was used. Synaptic membrane preparations (150 μg protein) were thawed and 5 μl was added to 20 μl buffer composed of 25 mM Tris/HCl, pH 7.4, 6 mM MgSO4, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol. In addition, for the detection of substrates for endogenous *c*AMP-dependent protein kinases, 2 μM *c*AMP and 1 mM isobutylmethylxanthine were added; alternately 1.5 mM $CaCl_2$ and 10 μM calmodulin were added for the detection of phosphorylation products by calmodulin-dependent protein kinase or 1.5 mM $CaCl_2$ and 50 μg/ml phosphatidylserine for protein kinase C-dependent phosphoproteins. After a preincubation of 90 sec at 38°C, the reaction was initiated by the addition of [γ-^{32}P]ATP (final concentration 2 μM, 1 μCi or 10,000 cpm/pmol) and allowed to proceed for 10 sec at 30°C and terminated by the addition of a cold solution containing 100 mM Tris/HCl, pH 8.6, 1 mM β-mercaptoethanol, 2% SDS, and 20% glycerol. The samples were boiled for 2 min and immediately frozen in liquid nitrogen for storage. 1–2-μl portions of the frozen samples were submitted to electrophoresis using a Pharmacia Phast-system. Two-dimensional electrophoresis was performed according to the procedure of BioRad using a Mini-Protean® apparatus (BioRad).

Plasma Membrane NADH-Dehydrogenase Activity

NADH-dehydrogenase activity in synaptic membrane preparations was determined spectrophotometrically according to the outlines of Crane and Loew (1976) and of Goldenberg *et al.* (1979) in 0.1 M potassium phosphate.

NADH-indophenol reductase (DCIP reductase) was determined with 0.13 mM NADH and 0.05 mM of 2,6 dichloro-indophenol added to 1.0 ml of the incubation mixture containing 50–200 μg membrane proteins in potassium phosphate buffer 0.1 M, pH 8.0, to start the reaction. Absorbency was recorded at 600 nm.

For ferricyanide reductase, the reaction mixture consisted of 0.1 M potassium phosphate, pH 7.0, 0.3 mM $K_3(Fe(CN)_6)$, 0.13 mM NADH, and enzyme (50–200 μg of protein). Ferricyanide reduction was recorded at 420 nm. Protein measurements on membrane vesicle fractions were by the biuret method.

Results and Discussion

ATP-Dependent Ca^{2+} Transport in Synaptic Membranes

The ATP-dependent uptake of $^{45}Ca^{2+}$ was linearly related to the amount of membrane protein in the assay. Time dependence studies of the uptake activity revealed that there was an initial influx of $^{45}Ca^{2+}$ which required about 4 min to reach equilibrium and decreased upon larger exposure (Fig. 1). All assay samples were therefore routinely preincubated for 4 min at 38°C prior to the addition of ATP, and the ATP-dependent Ca^{2+} transport was defined as the $^{45}Ca^{2+}$ taken up following the introduction of ATP. The kinetics and general characteristics of the Mg-dependent and ATP-driven Ca^{2+} transport observed in our preparations were in excellent agreement with the observations from Michaelis *et al.* (1987).

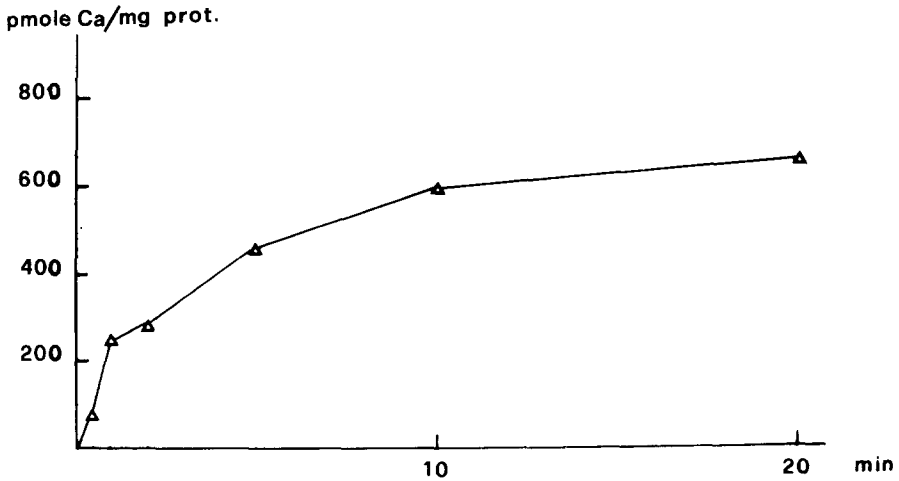


Fig. 1. Kinetics of ATP-dependent and ATP-independent Ca^{2+} accumulation into synaptic membranes. The Ca^{2+} uptake assays were performed under the conditions described in Material and Methods. The membranes were incubated for varying incubation periods at 35°C in media that contained 100 mM free Mg^{2+} and 100 mM ATP in the presence of 25 μg oligomycin, 0.1 mM ouabain, and 0.2 mM CDTA in 10 mM Tris-HCl, pH 7.0, and 100 mM KCl. The free Ca^{2+} concentration was held constant at 1 mM. Data are presented as percent of control experiments run in parallel without oxidizing (respectively, reducing) agents.

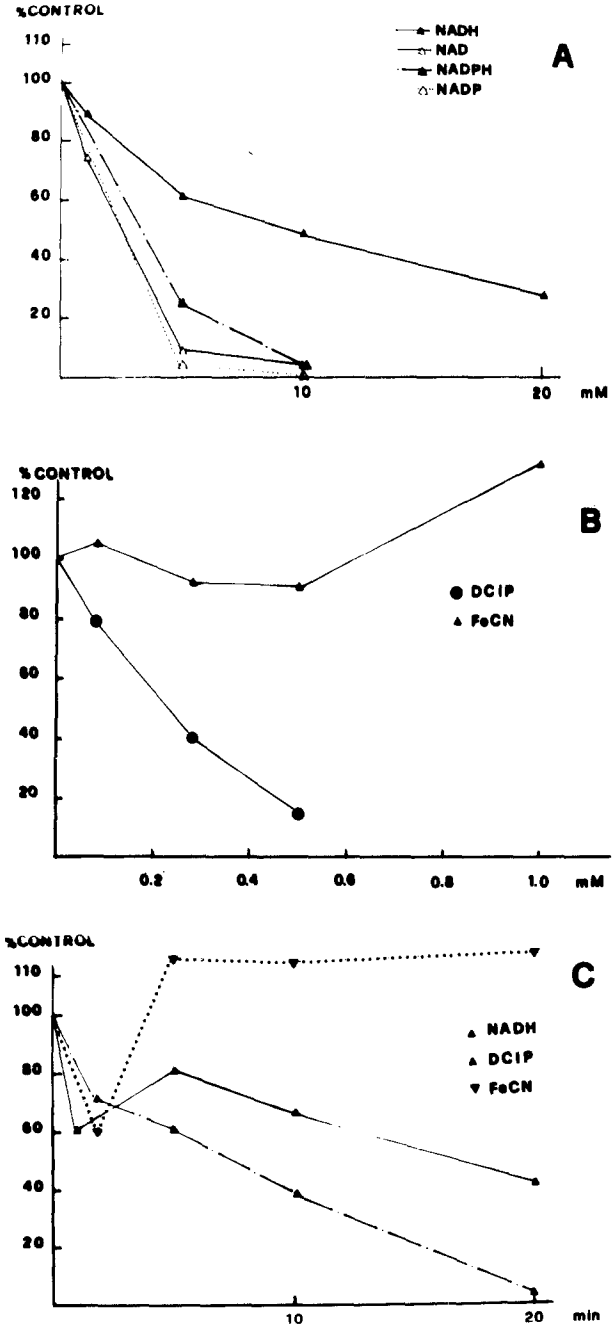
The effects of pyridine nucleotides on the synaptic ATP-dependent Ca^{2+} transport system were tested (Fig. 2). A complete inhibition of the Ca^{2+} transport system was observed at physiological concentrations of reduced pyridine nucleotides. The inhibition was very effective with IC_{50} of ca. 10 mM, 3 mM, 3.5 mM, and 3 mM for NADH, NAD, NADPH, and NADP, respectively (Fig. 2A). Oxidized pyridine nucleotides appeared to be more effective. The effect was very reproducible, but varied from time to time in its intensity, depending upon the preparation used. Pyridine nucleotides also decreased the nonspecific Ca^{2+} uptake (ATP-independent) into the synaptic vesicles by about 36% for NADH, 9% for NAD, 21% for NADPH, and 15% for NADP. Oxidizing agents such as DCIP also strongly inhibit the ATP-dependent Ca^{2+} transport, with an IC_{50} of ca. 0.2 mM (Fig. 2B). By contrast, the effects of ferricyanide were more difficult to assess with good reproducibility, probably due to its higher redox potential: Depending upon the material used for the assay, an inhibition was observed in several cases, but sometimes no effect or even a strong activation were observed. Since all pyridine nucleotides inhibit the ATP-dependent Ca^{2+}-transport, control experiments were run with adenosine and AMP, since they are part of the pyridine nucleotide molecule (Table I). Adenosine did not affect the ATP-dependent Ca^{2+}-transport, whereas AMP completely blocked this Ca^{2+}-efflux mechanism.

The inhibition of synaptic ATP-dependent transport system is time dependent, and complete inhibition requires ca. 20 min under the experimental conditions described in Material and Methods. Figure 2C shows

Table. I. Effects of Adenosine and AMP on Ca^{2+} Efflux Mechanisms

	Na^+/CA^{2+} exchanger	ATP-dependent Ca^{2+}-transport
Control	100	100
DCIP	75	0
NAD	82	3
NADP	16	0
NADH	62	35
NADPH	44	31
NADH-DCIP	45	0
NADPH-DCIP	42	0
Adenosine 1 mM	98	121
5 mM		100
AMP 1 mM	95	0
5 mM		0

[a] Residual Ca^{2+}-transport (respectively, exhange) is expressed in percent of control. Pyridine nucleotides were 5 mM, DCIP 0.5 mM, and Adenosine or AMP either 1 or 5 mM. The media and experimental procedures are as indicated in Materials and Methods.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time-dependence curves for ferricyanide, DCIP, or NADH. Similar inhibitions were obtained with the other redox agents discussed in this paper.

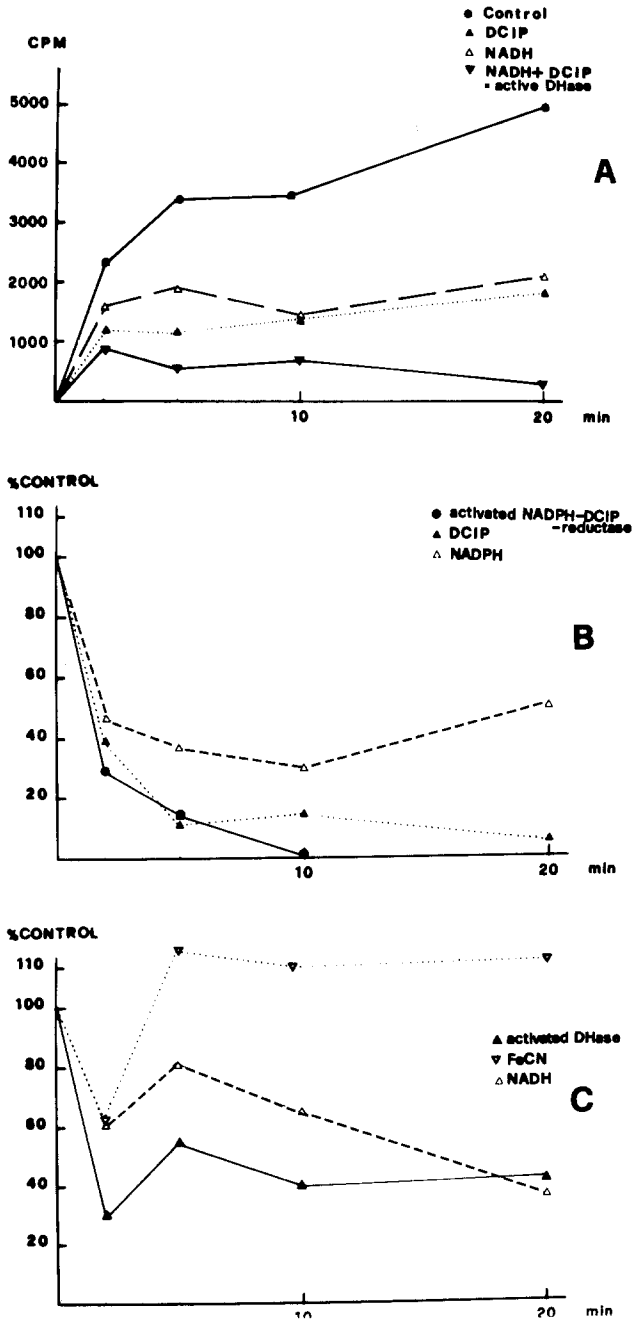
The inhibition of the ATP-dependent Ca^{2+} transport in synaptic vesicles by oxidizing and reducing agents was most effective when reduced NADH or NADPH was present together with an oxidizing agent such as either ferricyanide or DCIP (Fig. 3A–C), i.e., when the oxidoreductases of the synaptic plasma membranes had been fully activated by the presence of both substrates. These observations strongly suggest a key role of plasma membrane redox enzymes in controlling the Ca^{2+} export in synaptic nerve terminals.

Na^+/Ca^{2+} Exchange Mechanism

In a way similar to the studies described above, the effects of pyridine nucleotides and of oxidizing agents such as DCIP and ferricyanide have been tested on the Na^+–Ca^{2+} exchange mechanism (ATP-independent) of the same synaptic preparations. The results are presented in Figs. 4 and 5. As in the previous case, pyridine nucleotides inhibit the Na^+–Ca^{2+} exchange under similar conditions (Fig 5A, B), with IC_{50} values of ca. 8–10 mM for NAD and NADH and ca. 6 mM for NADP and NADPH. The effects are very reproducible and, again, in the absence of other oxidizing agents, the oxidized forms of the pyridine nucleotides appear more effective than the reduced ones. By contrast, no effect is observed with adenosine and adenosine monophosphate, both of which are constituents of the pyridine nucleotide molecule (Table I), again indicating that it is rather the nicotinamide moiety which is involved in the observed inhibition by pyridine nucleotides.

The inhibition by pyridine nucleoties is optimal with NADP at ca. 5 mM. Oxidizing agents such as DCIP and ferricyanide alone have little or no effects of the Na^+–Ca^{2+} exchange (Fig. 5C). When added together with reduced pyridine nucleotides, the inhibition produced by pyridine nucleotide was markedly reduced, by ca. 50% (Fig. 5D) with NADH as first cosubstrate, or unaffected (Fig. 5E) with NADPH as first cosubstrate, in contrast to the effects observed with the ATP-dependent Ca^{2+} transport system. This difference makes it nevertheless clear that the activation of plasma membrane

Fig. 2 Effects of various pyridine nucleotide and oxidizing agents on the Mg^{2+}-dependent, ATP-stimulated $^{45}Ca^{2+}$ transport in synaptic membranes. A and B: concentration dependence. Incubations were carried out for 10 min in the presence of either NADH, NAD, NADPH, NADP (A) or DCIP or ferricyanide (B) in addition to the buffer described in Materials and Methods. Pyridine nucleotides were 5 mM and the other oxidizing agents 0.7 or 1 mM, respectively, in the incubation buffer. (C) Time dependence curves of the influence of NADH and DCIP or ferricyanide on the Mg^{2+}-ATP-dependent Ca^{2+} transport system.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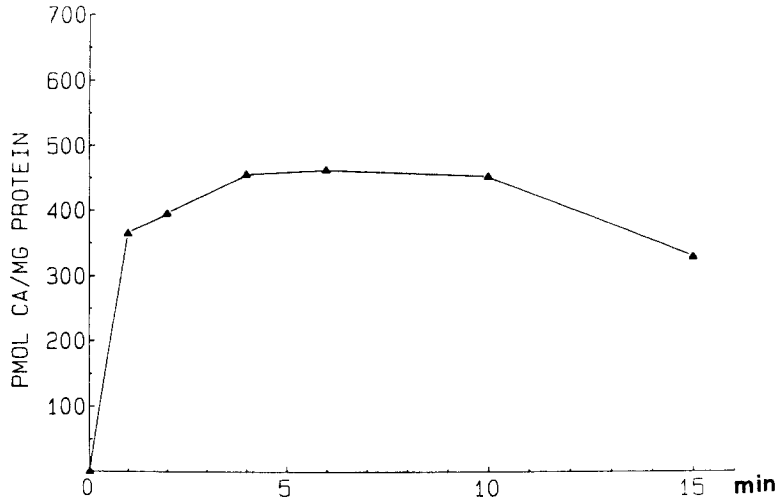

Fig. 4 Kinetics of the Na^+/Ca^{2+} exchange system in synaptic membranes. The assays were performed under the conditions described in Materials and Methods. The membranes were incubated for varying incubation periods at 35°C as indicated.

redox enzymes also modulates in some manner the pyridine nucleotide-mediated inhibition of the Na^+–Ca^{2+} exchange mechanisms in synaptic plasma membranes, allowing for a very subtle control of Ca^{2+} export by means of pyridine nucleotides.

Whereas dihydropyridine is known as an antagonist of Ca^{2+} channels responsible for Ca^{2+} entry into the cell, no pyridine analogue is known to be an antagonist of any of the transport systems responsible for Ca^{2+} export; so a direct action of the pyridine nucleotide molecule on the export systems themselves is very unlikely. The fact that the redox state of pyridine is apparently unimportant in this control process for Ca^{2+} export mechanisms is disturbing.

In the absence of a regulatory function of the pyridine nucleotide moiety at the level of the plasma membrane, it is possible that oxidized pyridine nucleotide is being rapidly reduced during the incubation period (which precludes a 4-min period of preincubation) by endogenous redox agents,

Fig. 3 Effects of NADH-DCIP and NADH-ferricyanide reductases on the Mg^{2+}-dependent, ATP-stimulated $^{45}Ca^{2+}$ transport in synaptic membranes. Synaptic redox enzymes were activated in the presence of 5 mM NADH and either 0.7 mM DCIP (A) or 5 mM NADPH and 0.7 mM (B) or 0.1 mM ferricyanide and 5 mM NADH (C). Various control experiments run in parallel are displayed also.

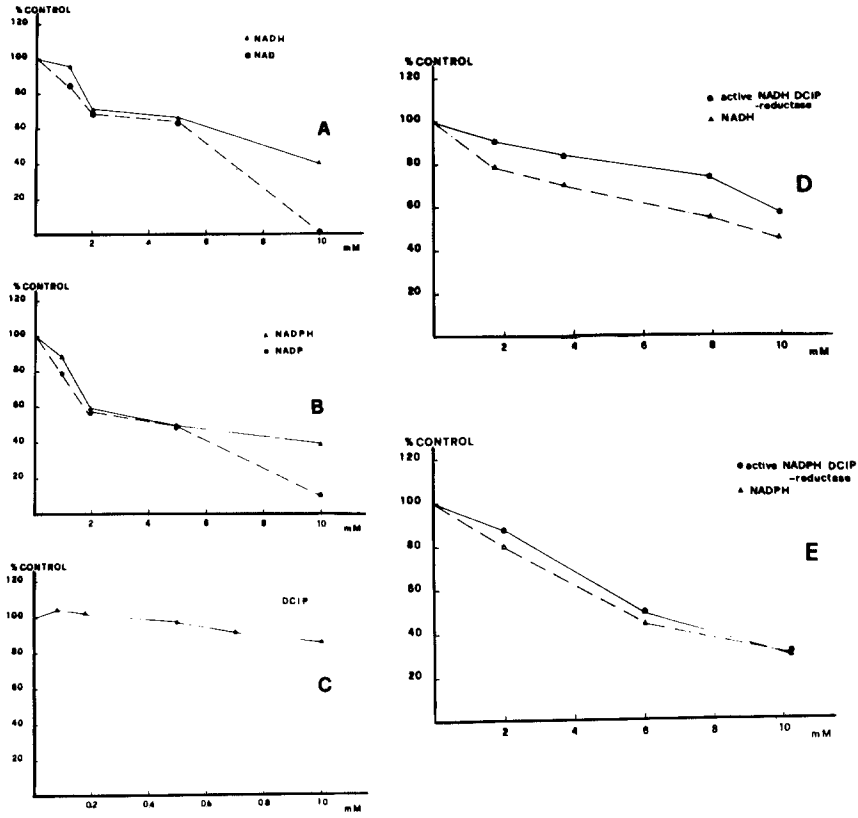

Fig. 5. Effects of pyridine nucleotides, DCIP, and of NADH-DCIP reductase on the Na^+/Ca^{2+} exchange in synaptic membranes. (A) Effects of various concentrations of NADH and NAD; (B) effects of NADPH and NADP; (C) effects of DCIP at various concentrations; (D) NADH-DCIP reductase was activated in the presence of 1 mM DCIP and variable amounts of NADH, up to 10 mM, as indicated in the figure. (E) Same as (D) but with variable concentrations of NADPH in the presence of 1 mM DCIP.

becoming available in their reduced forms as substrates for the NADH-dehydrogenases. To check this possibility, control experiments were run under experimental conditions similar to those of the Na^+/Ca^{2+}-exchange or of the ATP-dependent Ca^{2+}-transport system (Table II). Oxidized pyridine nucleotides added into the incubation media for these two Ca^{2+}-efflux systems are partly reduced within a few seconds after the addition. The reduction appears to be related to consumption of some endogenous substrate, since further addition of oxidized pyridine nucleotide is unaffected. The depletion of endogeneous compounds (or other processes) apparently makes some of the pyridine nucleotides available in their reduced form for the dehydro-

genases. The amount of endogenously generated NADH or NADPH could well account for the observed pyridine nucleotide-mediated inhibition of Ca^{2+}-efflux processes, at least to some extent; however, the involvement of other possibly related mechanisms is very possible and requires further studies.

On the basis of the characteristics described in this study, it appears clearly that transplasma membrane redox activity in synaptic vesicles strongly modulates both the ATP-driven Ca^{2+} transport and the Na^+/Ca^{2+} exchange mechanisms. Redox control of ion mobilization has been described in other studies. A meaningful correlation between plasma membrane oxidoreductase and Ca^{2+}-dependent K^+ transport activities has been presented (Miner *et al.*, 1983; Alvarez *et al.*, 1984; Garcia-Sancho *et al.*, 1979). Reducing agents loaded into red cell ghosts activate Ca^{2+}-dependent K^+ channels, a consequence of redox interactions connected with cellular function. Atebrin, a potent inhibitor of NADH-dehydrogenase (Crane *et al.*, 1989) activities of mammalian plasma membrane, inhibited the effect of electron donors on the K^+ channel, another evidence that these membrane-bound oxidoreductase systems could be involved. Together with our observations, these results emphasize the role of redox processes at the plasma membrane level.

Protein Phosphorylation

In view of the overall function of Ca^{2+} in the intracellular signal transduction, particularly in the activation of protein kinases C and Ca^{2+}-calmodulin-dependent protein kinases, the effects of the synaptic NADH-dehydrogenases on intrasynaptosomal protein phosphorylation were investi-

Table II. Reduction of Pyridine Nucleotides during Pre-incubation[a]

Additions	Na^+/Ca^{2+}-exchanger	ATP-dependent Ca^{2+}-transporter
NAD^+	80	401
$NADP^+$	48	50
Ca^{2+}-NAD^+	90	321
Ca^{2+}-$NADP^+$	50	241
Ca^{2+}-$NADP^+$-DCIP	450	240
NAD^+-DCIP	400	350
$NADP^+$-DCIP	430	1450

[a]Oxidized puridine nucleotide (5 mM) was added into the pre-incubation buffers for either the Na^+/Ca^{2+}-exchanger or the ATP-dependent Ca^{2+}-transporter (see Materials and Methods) in the presence of 100–200 μg synaptic plasma membranes. The amounts of reduced pyridine nucleotides formed during the pre-incubation period was followed spectrophotometrically at 340 nm and is presented (in nmol formed per mg of proteins).

gated. Two-dimensional electrophoresis was also used to help identify some of the phosphorylated proteins in either control experiments or in experiments made in the presence of reduced pyridine nucleotides and/or DCIP as substrates for activating synaptic plasma redox enzymes. As shown in Fig. 6, intrasynaptosomal protein phosphorylation is markedly affected by the activation of NADH-dehydrogenases. The data show that synaptic membrane redox systems are capable of completely inhibiting protein phosphorylation under physiological conditions. The effects are more pronounced in the presence of 1 mM DTT in the incubation medium (Table III), although the indophenol is being partly reduced under these conditions

Activation of *c*AMP-dependent protein kinases yielded five major phosphoproteins with MW of 22,000, 38,000, 54,000, 80,000 and 100,000 Da. In the presence of 1 mM NADH, the protein phosphorylation ratios were only 65, 30, 50, 70 and 80%, respectively, of its original value, whereas 1 mM NADPH had a weaker effect, reducing the phosphorylation ratio at 80, 65, 90, 90, and 95% of the values from the controls. DCIP did not affect the protein phosphorylation to a great extent either, yielding values between 10 and

Table III. Effects of Dithiothreitol on DCIP- and Pyridine Nucleotide-Mediated Inhibition of Intracellular Protein Phosphorylation[a]

Additions	A	B	C	D
Control	100	100	100	100
DTT 1 mM	97	90	83	87
DCIP, no DTT	91	78	35	44
DCIP and 1 mM DTT	82	24	19	24
NADH-DCIP, no DTT	35		58	70
NADH-DCIP, 1 mM DTT	10	21	3	41
NADP-DCIP, no DTT	30	92	43	
NADP-DCIP, 1 mM DTT	9	28	16	
NAD, no DTT	45	92	100	
NADP, no DTT	45	92	100	
NAD and DTT	28	67	36	
NADP and DTT	18	62	41	

[a]Synaptosomes were phosphorylated in the presence of [γ-^{32}P]ATP with the additions indicated, as described in Materials and Methods. Proteins were separated on SDA–8% polyacrylamide gels and phosphoproteins localized by autoradiography. (A) Effects on *c*AMP-dependent protein kinases with membranes incubated in the presence of 2 mM *c*AMP and 1 mM isobutylmethylxanthine in the buffer. (B) Effects on endogenous calmodulin-dependent protein kinases, tested in the presence of 1.5 mM $CaCl_2$ and 10 μg/ml calmodulin. (C) Effects on protein kinase C (tested in the presence of 1.5 mM $CaCl_2$ and 50 μg/ml phosphatidylserine in the buffer. (D) Effects on unspecific protein kinases. Other additions in each sample were as indicated. Pyridine nucleotides were 5 mM, DCIP 0.5 mM, and DTT 1 mM in the incubation buffer. See Materials and Methods for detailed experimental procedure. The inhibition of protein phosphorylation is expressed in percent of control experiments.

20% lower than the control experiments. However, when both substrates of plasma membrane oxidoreductases were added together, a very strong inhibition of intrasynaptosomal protein phosphorylation was repeatedly observed. Upon activation of NADH-DCIP-oxidoreductases, the inhibition affects virtually all phosphorylated proteins to the same extent, with the exception of the 38,000-Da protein which is less affected by NADH-DCIP-oxidoreductases. NADPH-DCIP-oxidoreductase also dramatically inhibits protein phosphorylation, yielding phosphorylation ratios less than 30% of the control experiments.

Ca^{2+}-calmodulin-dependent protein kinases, on the other hand, promotes the phosphorylation of seven major synaptic proteins with MW of ca. 25,000, 33,000, 40,000, 53,000, 80,000, 95,000, and 120,000 Da, respectively. In the presence of NADH or NADPH, the phosphorylation ratios of these proteins were little affected or somewhat enhanced (between 10 and 15%) over the ratios obtained in the absence of pyridine nucleotides. DCIP, when added alone, inhibited the Ca^{2+}-calmodulin-dependent phosphorylation by ca. 10–15% or even to ca. 76% in the presence of DTT (Table III). When both substrates of NADH-dehydrogenases were added, i.e., when synaptic plasma redox systems were fully active, an optimal inhibition of Ca^{2+}-calmodulin kinases was observed, resulting in virtually complete absence of protein phosphorylation with NADPH-DCIP-oxidoreductases and less than 20% phosphorylation ratios with NADH-DCIP-oxidoreductases.

Phosphorylation via protein kinase C (PKC) was also examined under appropriate conditions. In the absence of reducing or oxidizing agents, PKC induced the phosphorylation of eight major proteins with MW of ca. 25,800, 33,000, 50,000, 54,000, 61,000, 92,000, 108,000, 150,000 Da respectively. In the presence of 1 mM NADPH optimum phosphorylation ratios were obtained, yielding values 1.2- to 1.5-fold above the control experiments. By contrast, 1 mM NADH produced little effects on PKC, and the observed values corresponded almost to the control values. DCIP (0.3 mM) inhibited PKC, particularly in the presence of DTT or when added together with reduced pyridine nucleotides. Complete inhibition was observed with NADH-DCIP oxidoreductases, while NADPH-DCIP oxidoreductases decreased the intrasynaptosomal protein phosphorylation to ca. 16% of its control value.

The possibility of high concentrations (5 mM) of NAD^+ or $NADP^+$ blocking the protein phosphorylation patterns shown in Fig. 6 was also tested (see Table III), with a view of testing the possibility of pyridine nucleotides to function as competitive inhibitors of the substrate ATP. However, no inhibition is observed on Ca^{2+}-calmodulin-dependent or on protein kinase C- dependent protein phosphorylation (Table III) when NAD or NADP are added alone, i.e., in the absence of DTT as reductant or DCIP, while an inhibition was observed on *c*AMP-dependent protein phosphorylation. For

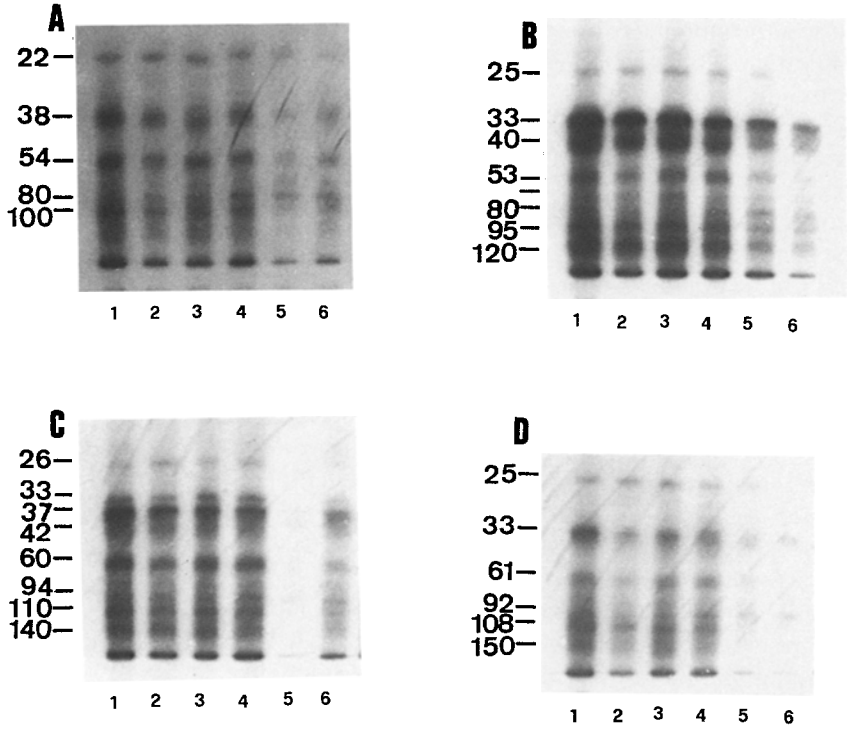

Fig. 6 Effects of synaptic plasma redox on the phosphorylation of protein in intact synaptosomes. Rat brain synaptosomes were prepared and phosphorylated in the presence of [γ-^{32}P]ATP with the additions indicated, as described in Materials and Methods. Proteins were separated on SDS–8% polyacrylamide gels and phosphoproteins localized by autoradiography. (A) Incubation for the detection of *c*AMP-dependent protein kinases proceeded in the presence of 2 μM *c*AMP, 1 mM isobutylmethylxanthine in the buffer made of 25 mM Tris-HCl, pH 7.4, 6 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol. (B) Incubation for the detection of substrates for endogenous calmodulin-dependent protein kinases was performed in 1.5 mM $CaCl_2$ and 10 μg/ml calmodulin in the same buffer. (C) Incubation for the detection of endogenous substrates for protein kinase C was performed in the presence of 1.5 mM $CaCl_2$ and 50 μg/ml phosphatidylserine in the same buffer. (D) Substrates for unspecific protein kinases were detected in the same buffer without addition. In all samples, 2 mM [γ-^{32}P]ATP (1 mCi per sample) was added to initiate the reaction. The reaction was allowed to proceed in a final volume of 30 μl with 100 μg proteins for 15 sec at 30°C and terminated by adding 15 μl of Laemmli sample buffer, boiled for 2 min at 100°C and stored in liquid nitrogen. Additions in each figure were as follows (from left to right): (1) control experiments with inactive synaptic oxidoreductases; (2) incubations performed in the presence of 5 mM NADH; (3) in the presence of 5 mM NADPH; (4) in the presence of 1 mM DCIP; (5) synaptic oxidoreductase fully activated in the presence of 5 mM NADH and 1 mM DCIP (= activated NADH-DCIP reductase); (6) same as (5) but with 5 mM NADPH and 1 mM DCIP (= activated NADPH-DCIP reductase).

this latter observation, further studies are necessary to delineate whether this effect is due to membrane redox enzymes in the synaptic plasma membrane, to competitive inhibition of adenylate cyclase itself, or to competitive inhibition of the substrate ATP on the protein phosphorylation step.

Conclusion

The salient features described in this paper are: (1) Ca^{2+} efflux from the synaptic plasma membrane is strongly inhibited by pyridine nucleotides, in the order NADP > NAD > NADPH > NADH for both extrusion mechanisms. The effects are specific and reproducible; (2) upon addition of the oxidizing agents tested, ferricyanide and DCIP, which both are substrates for synaptic plasma membrane dehydrogenases, the effects obseved with pyridine nucleotides alone were drastically changed: they could be partially reversed in the case of the Na^+–Ca^{2+} exchange mechanism, but the ATP-driven Ca^{2+} pump, by contrast, was further inhibited. In addition, synaptic plasma oxidoreductases also dramastically inhibit intracellular protein phosphorylation. Whether the latter effect is a consequence of the modulation of intracellular Ca^{2+} efflux remains to be established. Nevertheless it appears that synaptic redox enzymes dramatically control protein kinases and thus modulate intracellular protein phosphorylation by a yet unexplained mechanism. The significance of such a control mechanism remains to be established.

Acknowledgments

The authors are indepted to Chr. Poncet for invaluable technical assistance. The present work was supported by Grant No. 3.060.086 from the Swiss National Foundation.

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