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Biochimica et Biophysica Acta 1374 (1998) 34–46

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Sensitivity of the synaptic membrane Na^+/Ca^{2+} exchanger and the expressed NCX1 isoform to reactive oxygen species

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Received 2 February 1998; revised 22 June 1998; accepted 2 July 1998

Abstract

Two plasma membrane proteins, the Na^+/Ca^{2+} exchanger (NCX) and the Ca^{2+}-ATPase, are major regulators of free intraneuronal Ca^{2+} levels as they are responsible for extrusion of Ca^{2+} from the intracellular to the extracellular medium. Because disruption of cellular Ca^{2+} regulation plays a role in damage occurring under conditions of oxidative stress, studies were conducted to assess the sensitivity of the NCX to reactive oxygen species (ROS). Exchanger activity in brain synaptic plasma membranes and in transfected CHO-K1 cells was inhibited following brief exposure to the peroxyl radical generating azo initiator 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and to peroxynitrite. Incubation with hydrogen peroxide did not alter NCX activity, even at 800 μM concentration. In CHO-K1 cells transiently transfected with the NCX1 isoform of the exchanger, AAPH treatment decreased the maximal transport capacity (V_{max}), whereas the K_{act} remained unchanged. Peroxynitrite led to an increase in K_{act} with no change in V_{max}. Loss of activity following exposure to either AAPH or peroxynitrite was associated with the formation of high molecular weight aggregates of NCX, and AAPH also caused fragmentation of the exchanger protein. These findings suggest that the NCX is sensitive to biologically relevant ROS and could be involved in the loss of Ca^{2+} homeostasis observed under oxidative stress. 0005-2736/98/$ – see front matter

Keywords: Na^+/Ca^{2+} exchanger; Oxidative stress; Peroxyl radical; Peroxynitrite; Hydrogen peroxide

1. Introduction

The loss of cellular Ca^{2+} regulation, particularly in excitable cells such as myocytes and neurons, may be a common pathway leading to cell death [1,2], and Khachaturian and others have suggested that disruption of Ca^{2+} homeostasis may play a role in neurodegenerative changes underlying age-related dementia [3,4]. Although the mechanisms underlying altered Ca^{2+} regulation in aging brain are not known, it has been suggested that an age-related reduction in cellular antioxidant capacity would result in higher steady-state levels of free radicals, leading to inactivation of key cellular functions (e.g., [5,6]). Although reactive oxygen species (ROS) are ubiquitous in living organisms, elevated levels can lead to peroxidation of lipids, crosslinking of proteins, and DNA strand breaks [7]. Thus the Ca^{2+} transporting

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AEBSF, [4-(2-aminoethyl)benzenesulfonylfluoride, HCl]; CHO-K1, Chinese hamster ovary cell(s); DNPH, 2,4-dinitrophenylhydrazine; ROS, reactive oxygen species; SPM, synaptic plasma membrane

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00121-7

proteins located in the plasma membrane might be targets of ROS acting through effects on either membrane lipids or the proteins themselves. It has been shown, for example, that the lung endoplasmic reticulum Ca^{2+} pump is susceptible to ROS, and this is paralleled by inhibition of Ca^{2+} transport [8]. Furthermore, recent findings indicate that free radical-mediated damage to the plasma membrane leads to disruption of cellular Ca^{2+} homeostasis (e.g., [9,10]). With respect to neuronal viability, it has been shown that conditions leading to apoptosis as well as to necrosis are linked to intracellular Ca^{2+} accumulation and formation of ROS such as superoxide and peroxynitrite [11,12].

The Na^+/Ca^{2+} exchanger (NCX) is a major Ca^{2+} transporting protein located in the plasma membrane of all excitable and many non-excitable cells [13,14]. The exchanger translocates Ca^{2+} either into or out of the cytosol across the plasma membrane, depending on the Na^+ gradient [13]. ROS-mediated modification of the NCX protein in the plasma membrane could bring about altered Ca^{2+} homeostasis and compromised cell function. Some evidence exists indicating that the exchanger is sensitive to changes in redox conditions, but the results have been quite complex [15]. Nevertheless, studies with mammalian brain have shown sensitivity of the exchanger to anoxia [16], and experiments with intact myocytes indicated that exchanger current is sensitive to oxidative stress and that this inhibition of activity appears to involve sulfhydryl groups in the protein [17]. Given the important role that this transporter is believed to play in Ca^{2+} regulation, further exploration of the effects of oxidative stress on this system seems warranted.

During the past 6 years the exchanger has been cloned from different tissues resulting in the identification of genes encoding very similar proteins: NCX1 [18], NCX2 [19] and NCX3 [20]. Hydropathy profiles of the isoforms are very similar and postulate five putative transmembrane domains at the N terminus and six transmembrane domains at the C terminus, with a large intracellular loop in between, representing approx. 55% of the whole protein [18–20]. All three isoforms of the exchanger protein appear to be expressed in brain [20]. We have undertaken studies initially to assess the sensitivity of brain exchanger activity to in vitro oxidants. However, since it has already been demonstrated, for example, that specific isoforms of other membrane transporting proteins such as $(Na^+ + K^+)$-ATPase have differential sensitivities to in vitro oxidation [21,22], we decreased the complexity of our system by expressing a single isoform, NCX1, in CHO-K1 cells. Since this cell line does not normally express NCX genes [23], it was possible to transfect NCX1 into these cells and to examine the effects of oxidation on the activity of a protein with a fully defined primary structure.

2. Material and methods

2.1. Preparation of rat brain synaptic membranes

Synaptic plasma membranes (SPMs) were prepared from male Sprague-Dawley rats (3–6 months) as we have described previously [24,25]. The synaptosome-rich fraction was isolated at the interface of a discontinuous Ficoll gradient containing 8% and 14% Ficoll. Synaptosomes were lysed in 3 mM Tris-HCl, pH 8, and the synaptic membrane fraction was collected after centrifugation for 25 min at $31\,000 \times g$. The membranes were resuspended in 0.3 M sucrose, 50 μM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and stored in small aliquots at −70°C.

2.2. Cell culture of CHO-K1 and DNA transfection

CHO-K1 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in 90% Ham's F-12 medium and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) under 4.5% CO_2/95.5% air at 37°C. The cultures (25 cm^2 flasks) were passaged in a 1:4 split ratio every second day and used for transfection before they reached 20 passages. For the indicated studies designed to culture CHO-K1 cells at reduced CO_2 levels (0.04% CO_2), cells were maintained in 88% CO_2-independent medium (Gibco BRL, Gaithersburg, MD), 10% fetal bovine serum and 4 mM L-glutamine.

CHO-K1 cells were transfected with the mammalian expression vector pcDNA1 (Invitrogen, San Diego, CA) containing a 6 kb cDNA insert expressing the canine heart Na^+/Ca^{2+} exchanger, plasmid pcNCE6.0, described previously [26]. CHO-K1 cells were plated 22 h before transfection on 60 mm plas-

tic dishes at a density of $6–7\times10^5$. After the cells reached 60–70% confluence, they were transfected with 3 μg plasmid DNA and 18 μl lipofectamine (Gibco BRL) in 3 ml serum-free Ham's F-12 medium and incubated for 5 h at 37°C. After removal of the lipid-DNA complex-containing medium, the cells were incubated in 5 ml of standard culture medium for an additional 65 h. Control cells were transfected with the pcDNA1 vector without the NCX1 insert. The 65 h time point was experimentally determined to lead to maximal expression of Na^+/Ca^{2+} exchange activity, with consistent results for several transfected cell preparations.

2.3. Measurement of Na^+/Ca^{2+} exchange activity

Assay conditions for measuring the Na^+-dependent Ca^{2+} transport activity were optimized with respect to protein amount, incubation time at 30°C, and $CaCl_2$ concentration to obtain the conditions under which Ca^{2+} uptake was linear.

The SPMs were thawed rapidly in 150 mM sodium phosphate buffer, pH 7.4, for 6 min at 37°C to form Na^+-loaded membrane vesicles. The protein concentration of the samples was determined using the BCA assay (Pierce, Rockford, IL). Na^+-dependent Ca^{2+} transport activity was determined in SPM vesicles essentially as described previously [25,27]. Fifteen micrograms SPM protein were incubated in 500 μl of medium containing 160 mM KCl (or NaCl for no gradient condition), 10 mM Tris (pH 7.4 at 30°C), 10 μM $CaCl_2$ and 0.04 μCi/ml $^{45}CaCl_2$ for 30 s at 30°C. The uptake reaction was stopped by addition of 2.5 ml of ice-cold stop solution containing 160 mM KCl, 1 mM EGTA, 10 mM Tris-HCl at pH 7.4, and rapid filtration of the samples through a 0.45 μm membrane. Filters were washed once with stop solution and the retained radioactivity was counted.

Prior to the ^{45}Ca uptake assay the CHO-K1 cells were loaded with Na^+ by incubation in 1 mM ouabain for 50 min at 37°C on dishes before harvesting them. The cells were then scraped up and gently homogenized in 150 mM sodium phosphate buffer, pH 7.4. The protein concentration was determined as described above. The ^{45}Ca uptake in transfected CHO-K1 cells was determined by incubating 17 μg cell protein in 500 μl of the solutions indicated for SPMs plus 15 μM $CaCl_2$ (unless indicated otherwise), 1 mM ouabain and 0.5 μCi/ml $^{45}CaCl_2$ for 20 s at 30°C.

For all transport assays the Na^+-dependent ^{45}Ca uptake was calculated by subtracting the ^{45}Ca that was bound or taken up in the absence of a Na^+ gradient from the transport in the presence of a Na^+ gradient [25,27]. The data were analyzed using non-linear curve fitting (Sigma Plot) to determine K_{act} and V_{max} based on the Michaelis-Menten equation. Statistical analysis of differences between samples was carried out using *t*-tests for unpaired samples.

2.4. Exposure of synaptic membranes and transfected CHO-K1 to in vitro oxidants

Exposure of the SPMs or cell suspensions to the oxidants was carried out at protein concentrations of 2 μg/μl in 150 mM sodium phosphate buffer at pH 7.4. The hydrophilic azo initiator 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) was used to generate peroxyl radicals by thermal decomposition [28]. Samples were exposed to AAPH for 15 min at 37°C, placed on ice, and the assays performed as indicated. The AAPH stock solution (0.5 M) was diluted in doubly distilled water to achieve the final concentrations indicated.

Exposure of SPMs and transfected CHO-K1 cells to hydrogen peroxide (H_2O_2) was similar to the oxidation experiments with AAPH. A 30% H_2O_2 stock solution was freshly diluted 1/1000 with doubly distilled water and added to the samples to achieve the final concentrations indicated. Tissue samples were incubated with H_2O_2 for 10 min at 37°C, and the oxidation reaction was terminated with 1 unit catalase (Boehringer Mannheim, Indianapolis, IN) per 1 μmol H_2O_2.

Peroxynitrite was synthesized by the reaction of ozone with ice-cooled aqueous azide at pH 12 as described [29]. Prior to the actual oxidation experiments, the concentration of peroxynitrite was estimated spectrophotometrically at 302 nm using $\varepsilon_{302nm} = 1670\ M^{-1}\ cm^{-1}$ [30]. Stock solutions of 60 mM peroxynitrite were diluted with alkaline water (pH 12) to achieve 20 mM. Aliquots of this diluted peroxynitrite solution were added to the protein samples to achieve the final concentrations indicated. Peroxynitrite was added slowly using a 5 μl micro-

syringe (Hamilton, Reno, NV) because of the fast decomposition kinetics of peroxynitrite at high buffer concentrations. The described experimental approach ensures a more homogeneous distribution of peroxynitrite in the solution [31]. Samples were incubated for 5 min at room temperature. A reversed-order-of-addition control experiment with peroxynitrite was conducted to determine the effect of decomposition products of peroxynitrite, i.e., nitrate and nitrite, on the protein sample. The control experiment was conducted in the following way: peroxynitrite was added to 150 mM sodium phosphate buffer, pH 7.4 at a final concentration of 400 μM, and the peroxynitrite was allowed to decompose before the protein sample was added to the reaction mixture after 5 min (reversed-order-of-addition). The ^{45}Ca uptake activity in these samples was essentially the same as that in untreated protein preparations.

2.5. Electrophoresis and immunoblot analysis

Transfected CHO-K1 cells were homogenized in 1% SDS, 0.32 M sucrose, 1 mM [4-(2-aminoethyl)-benzenesulfonylfluoride, HCl] (AEBSF) (Calbiochem, La Jolla, CA), 0.1 mM EDTA and 10 mM potassium phosphate buffer, pH 7.4. Indicated amounts of protein were diluted in SDS sample buffer (10% glycerol, 2% SDS, 0.05% bromophenol blue, 50 mM Tris-HCl, pH 6.8) in the absence of 2-mercaptoethanol without heating (non-reducing conditions) or in the presence of 2.5% 2-mercaptoethanol (reducing conditions). Proteins were separated on 5 or 7% gels and, after electrophoretic separation, the proteins were transferred to PVDF membranes by electroblotting using standard conditions.

The NCX protein was detected using the rabbit antisera for this protein at 1/400 dilution (Swant, Bellinzona, Switzerland). Nitrotyrosine formation was probed using rabbit polyclonal anti-nitrotyrosine antibodies diluted 1/1500 (Upstate Biotechnology, Lake Placid, NY), and the 2,4-dinitrophenylhydrazine (DNPH)-derivatized carbonyls were detected using the rabbit polyclonal anti-DNPH antibodies diluted 1/1000 (Sigma, St. Louis, MO). Detection was achieved using a goat anti-rabbit IgG alkaline phosphatase conjugate from Zymed (San Francisco, CA) or goat anti-mouse IgG alkaline phosphatase conjugate from Pierce (Rockford, IL). After washing with PBS containing 0.05% Tween 20, the substrate solution (0.1 mg/ml nitroblue tetrazolium, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 4 mM $MgCl_2$, 0.1 M Tris, pH 9.8) was added and the reaction allowed to proceed usually for about 2–3 min.

In experiments designed to probe for the formation of carbonyls, the SPMs or transfected CHO-K1 homogenates were derivatized with DNPH according to Shacter et al. [32]. As a positive control, carbonylated bovine serum albumin (BSA) was generated by derivatizing 1 mg BSA with 1 μl of 1-bromo-2-butanone in a total volume of 1 ml of 20 mM sodium phosphate buffer, pH 7.9, for 3 h at room temperature (E. Floor, personal communication). The derivatization was monitored spectrophotometrically based on the maximum absorbance (360–390 nm) using a molar absorption coefficient of 22 000 M^{-1} cm^{-1} [33]. Derivatized protein samples were separated on a 7% SDS-polyacrylamide gel, electroblotted, and probed as described above.

3. Results

3.1. Effect of oxidants on Na^+/Ca^{2+} exchanger activity in rat brain SPMs

In an initial series of experiments, we investigated the sensitivity of the SPM Na^+/Ca^{2+} exchanger to in vitro exposure to various oxidants. The hydrophilic azo initiator AAPH generates peroxyl radicals in the aqueous phase by thermal decomposition [28], making it possible to produce peroxyl radicals likely to interact initially with regions of protein extending beyond the membrane bilayer. The effect of exposing SPMs to μM concentrations of peroxyl radicals on the Na^+-dependent ^{45}Ca uptake is shown in Fig. 1. Using AAPH as the oxidant, it is possible to calculate the rate of free radical generation (R_i) for the aqueous phase of the solution [28]. As indicated in Fig. 1, concentrations of AAPH ranging from 5 to 100 mM would produce peroxyl radical concentrations ranging from 6 to 122 μM under the incubation conditions used in these studies. The level of free radical generation, C_i, from decomposition of AAPH at 37°C can be calculated as follows:

$$C_i = 2eR^{\cdot}[\mathrm{AAPH}] \times t = 1.36 \times 10^{-6}\ s^{-1} \times [\mathrm{AAPH}] \times t$$

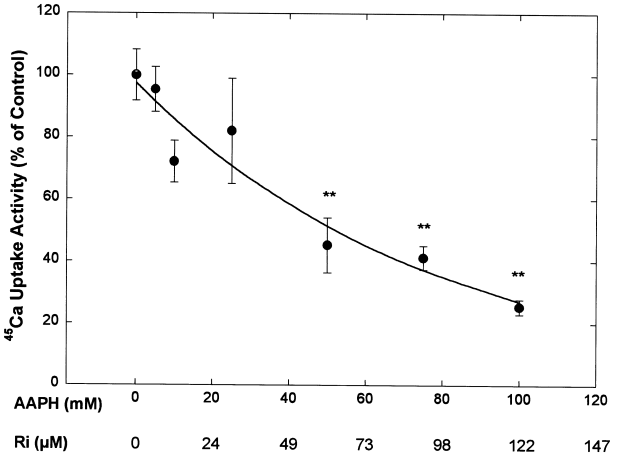

Fig. 1. Effects of increasing concentrations of AAPH on rat brain SPM Na^+/Ca^{2+} exchanger activity. SPMs were exposed to 5, 10, 25, 50, 75 and 100 mM AAPH under conditions calculated to generate peroxyl radical concentrations (R_i) of 6, 12, 31, 61, 92 and 122 μM R_i. The control samples were incubated under the same conditions, but AAPH was replaced with H_2O. The control activity was 1.71 ± 0.08 ($n = 6$). Data points are means ± S.E.M. of six to nine determinations. Samples that differed significantly from controls are indicated, $P = 0.01$ (**).

where e is the efficiency of free radical production and the concentration of AAPH is in moles/liter [28]. Increasing concentrations of AAPH caused progressive loss of the Na^+-dependent ^{45}Ca uptake activity of the synaptic membranes. At the highest AAPH concentration corresponding to 122 μM R_i produced, we observed a 74% reduction in activity. Non-linear curve fitting of the data indicated the IC_{50} for R_i to be approx. 66 μM. It is important to note that about 45% of the Na^+/Ca^{2+} exchanger protein is localized within the membrane and is not readily accessible to radicals produced in the aqueous phase. However, the peroxyl radicals in the aqueous phase can also induce lipid oxidation within the bilayer.

While oxidants such as azo initiators or light-activated sources of ROS provide a somewhat controlled means of generating free radicals, these sources are not physiological. As indicated in preceding sections, stimulation of neuronal receptors that cause increases in intracellular $[Ca^{2+}]_{free}$ also enhanced the formation of ROS species such as superoxide ($^{\cdot}O_2^-$), nitric oxide ($^{\cdot}NO$), and the reaction product between these two radicals, peroxynitrite ($ONOO^-$) [34]. Peroxynitrite is a strong oxidizing agent, but one that reacts rather selectively with certain proteins. Exposure of rat brain SPMs to peroxynitrite led to a concentration-dependent decrease in the Na^+/Ca^{2+} exchanger activity (Fig. 2). A maximal inhibition of 40% of the transport activity occurred at about 150 μM peroxynitrite, and no further reduction was observed with peroxynitrite concentrations up to 400 μM (Fig. 2). Control samples contained 400 μM decomposed peroxynitrite (reversed-order-of-addition). The failure of higher concentrations of peroxynitrite to reduce exchanger activity by more than 40% suggests that the NCX protein in synaptic membranes has a limited number of cysteines and tyrosines, the residues with which the peroxynitrite most readily reacts. Since it has recently been demonstrated that peroxynitrite rapidly permeates phospholipid membranes [35], it is likely that all such reactive sites were available to this reagent.

Another strongly oxidizing ROS species formed in cells from the dismutation of $^{\cdot}O_2^-$ is H_2O_2. A 10 min exposure (37°C) of SPMs to H_2O_2 at concentrations ranging from 100 to 800 μM produced no alteration in the Na^+-dependent ^{45}Ca uptake. Apparently, H_2O_2 is not reactive enough to cause H_2O_2-induced activity loss of the Na^+/Ca^{2+} exchanger under our experimental conditions (data not shown).

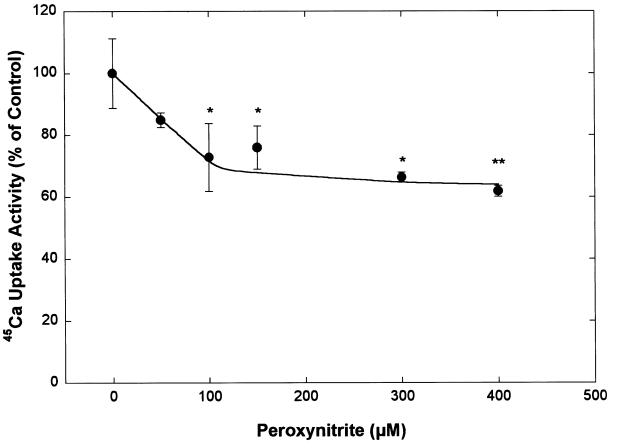

Fig. 2. Effects of increasing concentrations of peroxynitrite on rat brain SPM Na^+/Ca^{2+} exchanger activity. SPMs were exposed to 50, 100, 150, 300 and 400 μM peroxynitrite. The control samples represent the reversed-order-of-addition experiment as described. The exchanger activity in the controls was 2.09 ± 0.11 ($n = 6$). Data points are means ± S.E.M. of six to nine determinations. Samples that differed significantly from controls are indicated, $P < 0.05$ (*) and $P = 0.01$ (**).

3.2. Effect of oxidants on the activity of the NCX1 isoform expressed in CHO-K1 cells

Since rat brain SPMs express all three isoforms of the Na^+/Ca^{2+} exchanger, it seemed that more detailed studies on the effects of in vitro oxidants should be carried out with a simpler Na^+/Ca^{2+} exchanger system. Thus, we expressed the NCX1 isoform in CHO-K1 cells, a cell line shown to have no endogenous Na^+/Ca^{2+} exchange activity or protein expression [23]. We initially confirmed earlier reports that no exchanger activity could be determined in CHO-K1 cells transfected only with the pcDNA1 vector. This transfected CHO-K1 system permitted us to conduct some initial explorations of the potential molecular events underlying the inhibitory effects of the oxidants on a single well-characterized form of the exchanger protein.

Similar to the results obtained with rat brain synaptic membranes, exposure of the expressed NCX1 isoform to AAPH decreased ^{45}Ca uptake with increasing AAPH concentrations (Fig. 3A). At 100 mM AAPH, i.e., 122 μM R_i, 80% of the ^{45}Ca uptake was lost. For the oxidation of the NCX1 isoform with AAPH we calculated an IC_{50} value of 38.4 mM AAPH corresponding to 47 μM R_i. The effect of AAPH oxidation on the Ca^{2+} transport activity of the NCX1 isoform was further investigated to determine whether the peroxyl radicals were affecting the affinity of the NCX1 for Ca^{2+}, the V_{max} for transport or both. Transfected CHO-K1 cells oxidized with 28 mM AAPH (34 μM R_i), corresponding to approx. 40% inhibition of the NCX1 activity (see Fig. 3A) were compared to untreated transfected CHO-K1 cells in the Ca^{2+} transport assay at increasing $CaCl_2$ concentrations (Fig. 3B). Untreated CHO-K1 cells showed a maximal transport activity (V_{max}) of 1.1 nmol/mg with a K_{act} of 10.8 μM. CHO-K1 cells treated with AAPH exhibited a maximal transport activity that was half of the control value ($V_{max} = 0.6$ nmol/mg) with a K_{act} of 11.7 μM. This result suggests that the maximal transport activity was primarily affected by the oxidizing conditions whereas the affinity of the NCX1 isoform for Ca^{2+} remained essentially unchanged.

The treatment of transfected CHO-K1 cells with increasing concentrations of peroxynitrite inhibited the NCX1 activity in a concentration-dependent

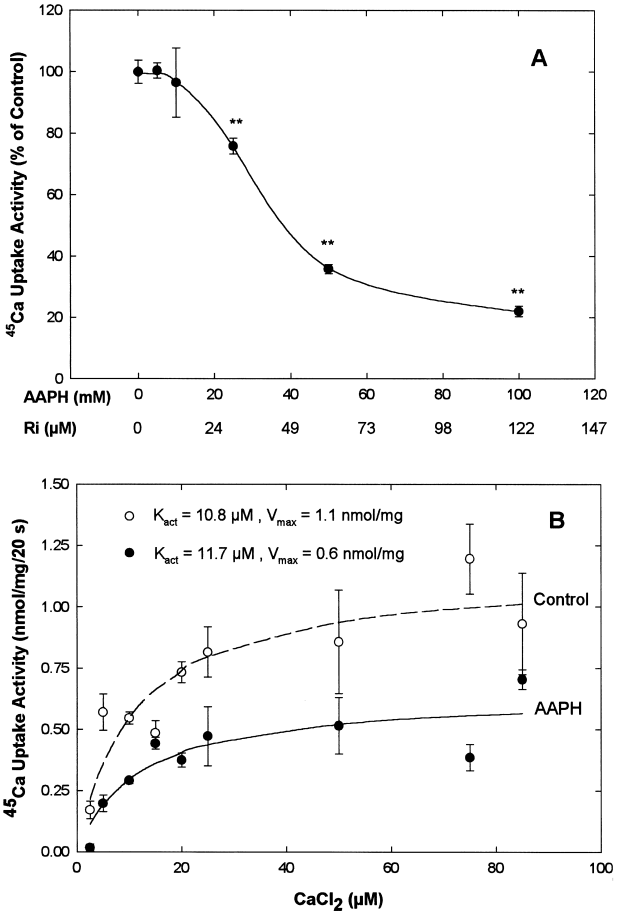

Fig. 3. Effects of AAPH on the Na^+/Ca^{2+} exchanger activity in CHO-K1 cells expressing the NCX1 isoform. (A) Cells were exposed to the indicated concentrations of AAPH as described in the legend for Fig. 1. The control activity was 0.54 ± 0.03 ($n = 6$). Data points are means ± S.E.M. of six determinations from two independent transfections. Samples that differed significantly from controls are indicated, $P = 0.01$ (**). (B) Exchanger activity was measured in CHO-K1 cells as a function of $[Ca^{2+}]$ in the presence (●) or absence (○) of 28 mM AAPH (34 μM R_i). Data points are means ± S.E.M. of nine determinations from three independent transfections. The calculated V_{max} values for AAPH-exposed samples and controls differ at $P < 0.05$.

manner. As was observed with the synaptic membranes, the exchanger activity could be decreased only to approx. 50% of the control value (reversed-order-of-addition). A plateau in the inhibition of exchanger activity was reached at concentrations greater than 150 μM peroxynitrite (Fig. 4A). In order to examine the nature of the inhibition by peroxynitrite, we measured the effect of this compound on the transport activity as a function of $[Ca^{2+}]$ by exposing

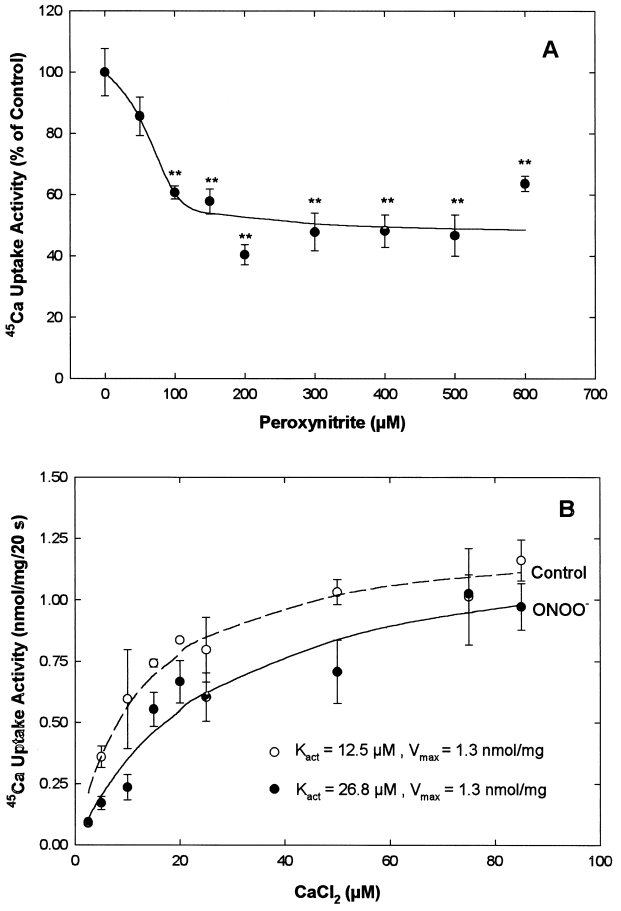

Fig. 4. Effects of peroxynitrite on the Na^+/Ca^{2+} exchanger activity in CHO-K1 cells expressing the NCX1 isoform. (A) Cells were exposed to 50, 100, 150, 200, 300, 400, 500 and 600 μM peroxynitrite. The control samples represent the reversed-order-of-addition experiment and the activity was 0.7 ± 0.08 ($n = 6$). Data points are means ± S.E.M. of six determinations from two independent transfections. Samples that differed significantly from controls are indicated, $P < 0.01$ (**). (B) Exchanger activity was measured in CHO-K1 cells as a function of $[Ca^{2+}]$ in the presence (●) or absence (○) of 150 μM peroxynitrite. The control represents the reversed-order-of-addition experiment with 150 μM decomposed peroxynitrite. Data points are means ± S.E.M. of nine determinations from three independent transfections. The calculated K_{act} values for peroxynitrite-exposed samples and controls differed at $P < 0.05$.

the vesicles of transfected CHO-K1 cells to 150 μM peroxynitrite and determining the maximal transport activity V_{max} and K_{act} (Fig. 4B). The V_{max} of the peroxynitrite treated cells ($V_{max} = 1.3$ nmol/mg) did not differ from that of untreated cells ($V_{max} = 1.3$ nmol/mg). However, the K_{act} of cells exposed to 150 μM peroxynitrite was more than double (26.8 μM) that of control cells (12.5 μM). Thus, it seems that the predominant effect of peroxynitrite was on the affinity of the exchanger for Ca^{2+}, and that the increase in K_{act} by this oxidant led to substantially lower transport at the lower Ca^{2+} concentrations. Following peroxynitrite treatment, transport activity measured at 15 μM Ca^{2+} was about 40% below that in untreated cells (Fig. 4B). This is consistent with the 40% reduction in transport activity at 15 μM Ca^{2+} following exposure to 150 μM peroxynitrite as shown in Fig. 4A. The results of these experiments suggest that peroxynitrite selectively modifies the NCX protein near the binding site for Ca^{2+}, but this modification has no effect on the maximal rate of Ca^{2+} transport.

The results from experiments in which the transfected CHO-K1 cells were exposed to H_2O_2 concentrations ranging from 50 to 800 μM revealed the same lack of effect on the exchanger activity as that determined for SPMs. None of the H_2O_2 concentrations used produced any detectable inhibition of the activity of the expressed NCX1 isoform (data not shown).

3.3. *Effect of oxidants on the structure of the NCX protein*

In efforts to determine whether the decrease in the exchanger activity after exposure of transfected CHO-K1 cells and SPMs to AAPH and peroxynitrite was correlated with detectable structural changes in the protein, we carried out experiments to probe for the formation of aggregates, evidence of fragmentation, formation of nitrotyrosine, or enhanced levels of carbonyl groups. Structural changes were investigated at oxidant concentrations at which approx. 50% of the NCX1 activity was lost when compared to the controls.

Fig. 5A displays a Coomassie blue-stained gel (5%) run under non-reducing conditions to reveal the presence of protein aggregates that might have resulted from the crosslinking of some proteins (indicated by arrow). Transfected CHO-K1 cells were oxidized with 800 μM H_2O_2 (lane 1), 400 μM peroxynitrite (lane 2) or with 50 mM AAPH corresponding to 61 μM R_i (lane 3), and compared to control cells (lane 4). It does appear that exposure of cells to H_2O_2, AAPH, and peroxynitrite led to accumulation of large molecular size protein species, presumably ag-

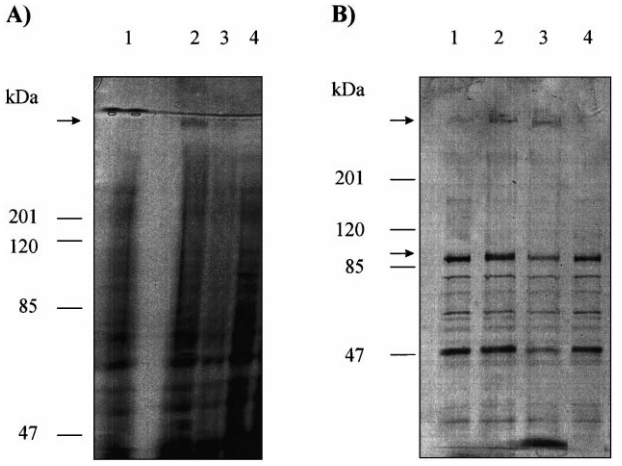

Fig. 5. Effects of AAPH, peroxynitrite and H_2O_2 on aggregation/fragmentation of the expressed NCX1 isoform. Transfected cells were exposed to 800 μM H_2O_2 (lane 1), 400 μM peroxynitrite (lane 2) or 50 mM AAPH corresponding to 61 μM R_i (lane 3). Control cells were similarly incubated, but the oxidant was replaced with H_2O (lane 4). 160 μg protein (16×20 cm gel, panel A) or 50 μg protein (7.5×10 cm gel, panel B) were subjected to electrophoresis on a 5 or 7% Laemmli gel, respectively, and proteins were either stained with Coomassie Blue (panel A) or probed with the rabbit antisera for Na^+/Ca^{2+} exchanger (panel B). The positions of the molecular mass markers are indicated on the left.

gregates, that did not penetrate the gel. Fig. 5B shows a Western blot after hybridization with the anti-Na^+/Ca^{2+} exchanger antibody following transfer of the proteins from a 7% gel also run under non-reducing conditions.

The polypeptide pattern of the immunodetected NCX1 with a deduced protein mass of 108 kDa (Fig. 5B, control lane 4) is similar to that observed after expression of the same cDNA clone in human embryonic kidney cells [26]. Detection of one major band near 108 kDa differs from the pattern described by Gabellini et al. [26], in which at least three species in the 120 kDa region were resolved. This could be due to glycosylation and/or posttranslational modifications that differ in the two cell lines [23]. In control cells the exchanger antibodies also labeled two polypeptides with molecular masses of approx. 80 and 70 kDa that were more lightly stained and one species of 50 kDa that showed nearly the same intensity as the 108 kDa band (Fig. 5B, control lane 4). According to Nicoll et al. [36] the 70 kDa band is assumed to arise from proteolysis of the 108 kDa protein, and this band was consistently observed in SPMs as well. The band detected at 80 kDa could be caused by degradation of misinserted overexpressed polypeptides [26]. The presence of a band at 50 kDa has not been reported in the literature; however, it might be a cell-specific degradation product of the expressed NCX1, as this band was not observed in the SPMs.

Incubation of both SPMs and transfected CHO-K1 cells with either AAPH or peroxynitrite led to aggregation of NCX1, as indicated by the formation of high molecular weight species during gel electrophoretic separation under non-reducing conditions shown for the CHO-K1 cells in Fig. 5B (lanes 2 and 3). No immunoreactive aggregates were observed in the control cells (lane 4) or following exposure to H_2O_2 (lane 1), an observation consistent with the lack of effects on exchanger activity. When SDS-PAGE was carried out in the presence of 2-mercaptoethanol (reducing conditions), densitometric analysis indicated that aggregate formation following treatment with 50 μM peroxynitrite was almost fully reversed, whereas about 10% of the original aggregate formation remained in the membranes exposed to 400 μM peroxynitrite (data not shown). When the membrane vesicles were exposed to AAPH (approx. 30 μM peroxyl radical concentration), reducing conditions led to a reversal of 90% of the aggregate formation, whereas vesicles exposed to the 60 μM peroxyl radical concentrations showed only about a 10% reversal (data not shown). These observations suggest that the aggregates are formed partially via intermolecular disulfide bridges. Other investigators have reported findings that would support the involvement of thiol modification in Na^+/Ca^{2+} exchange activity under conditions of oxidative stress [15,17]. For example, it was shown that sulfhydryl modifying agents such as diamide [17] and reduced-oxidized glutathione [15] are capable of modifying Na^+/Ca^{2+} exchanger activity.

It is worth noting that AAPH also caused substantial fragmentation of the expressed NCX1 isoform, which was detectable as decreased staining of NCX1-related bands and the appearance of smaller proteolytic products close to the dye front (Fig. 5B, lane 3). This observation suggested that random fragmentation of NCX1 occurred as a result of AAPH treatment with no major product of a specific size. A similar observation was described for fibronectin

after oxidation with Fe-EDTA, H_2O_2, and ascorbate [37].

Nitrotyrosine is a common product of peroxynitrite-mediated nitration at the *ortho* position of tyrosine residues, and there is evidence that CO_2 catalyzes the peroxynitrite-mediated nitration of tyrosine residues [38–40]. Therefore, the CHO-K1 cells were maintained and transfected with the plasmid pcNCE6.0 expressing the NCX1 isoform in CO_2-independent medium under air containing only 0.04% CO_2. Western blot analysis of the peroxynitrite-treated cells did not show the formation of nitrotyrosine in bands corresponding to the NCX protein. However, since the cell preparations contain a large number of proteins, only a small fraction of which represents the exchanger, the absence of any reactivity with the anti-nitrotyrosine antibodies could be due to limited sensitivity of the assay. When SPMs were analyzed for reactivity with anti-nitrotyrosine antibodies, two bands at approx. 80 and 130 kDa were observed repeatedly in control membranes. Exposure of SPMs to 400 μM peroxynitrite led to the appearance of many immunoreactive bands, and it was not possible to determine whether the NCX proteins were among those labeled. In efforts to determine whether the NCX protein was labeled by the antibodies, we solubilized the SPMs, partially purified the protein by immunoextraction with NCX antibodies, and analyzed this extract for the presence of nitration on the NCX protein bands. The protein from control membranes showed no reactivity with the anti-nitrotyrosine antibodies, whereas the protein extracted from membranes exposed to 400 μM peroxynitrite showed very faint but specific labeling of a band at approx. 70 kDa (data not shown). This same band was strongly labeled by the NCX antibodies, suggesting that the NCX protein may become nitrated in the presence of this agent. The level of nitration appears to be quite low as would be expected for an agent that reacts selectively with a limited number of residues.

The SPMs and transfected CHO-K1 cells were examined for the presence of an increase in carbonyls following exposure to 50 mM AAPH, 400 μM peroxynitrite, and 800 μM H_2O_2. Immunoblot analyses of DNPH-derivatized protein samples revealed the presence of numerous immunoreactive bands in control samples, an observation consistent with other reports [6,41]. Exposure to AAPH increased the intensity of labeling of several bands, but the peroxynitrite and H_2O_2 increased the labeling only slightly. Since it was not possible to identify the NCX proteins in these preparations, we again used the immunoextraction procedure and tested for the presence of enhanced carbonyl formation in protein extracted from membranes exposed to the oxidants. The approx. 70 kDa band observed in the immunoextracted fractions did show slightly enhanced reactivity with the anti-DNPH antibodies, and this was particularly prominent following exposure to peroxynitrite. These results indicate that in vitro exposure to oxidants leads to increased carbonyl formation on the NCX, though the nature of the reactive sites is not known.

4. Discussion

There is substantial evidence to suggest that aging in the brain is associated with altered intracellular Ca^{2+} regulation [3,4], and enhanced oxidative stress may play a role in the cellular mechanisms underlying age-related alterations [5,6]. Thus it is possible that modifications in Ca^{2+}-regulating proteins occur under conditions of oxidative stress, and such modifications may contribute to the observed alterations in Ca^{2+} regulation that occur with aging in brain neurons. Since the Na^+/Ca^{2+} exchanger represents one important plasma membrane Ca^{2+} regulating protein that exhibits decreased affinity for Ca^{2+} in aged brain membranes [27], these studies were carried out to assess the sensitivity of this Ca^{2+} transporter to various oxidants. After determining that exchanger activity in SPMs was indeed sensitive to inhibition by in vitro oxidants, one of the three known isoforms was expressed in a cell line devoid of endogenous exchanger protein as a first step in probing the specific regions of the exchanger molecule that are susceptible to modification by ROS.

Both the SPM Na^+/Ca^{2+} transporter and the expressed NCX1 isoform were found to be sensitive to peroxyl radicals generated by AAPH and to peroxynitrite but not to H_2O_2, even at a concentration of 800 μM. This observation is similar to that reported by Reeves et al. [15], showing that H_2O_2 concentrations up to 1 mM had no effect on the Na^+/Ca^{2+} exchanger activity in bovine cardiac sarcolemmal

vesicles. The possibility of enhancing the oxidative effects of H_2O_2 by increasing the incubation time of the reaction mixture at 37°C according to Huang et al. [21] could not be readily explored as prolonged 37°C incubation of our preparations decreased the Na^+-dependent ^{45}Ca uptake even in the absence of H_2O_2.

In contrast to H_2O_2, exposure to the oxidant AAPH resulted in a reduction in the ^{45}Ca uptake activity by both SPMs and the expressed NCX1 isoform (Figs. 1 and 3A), with the primary effect being a decrease in the V_{max} (Fig. 3B). Since AAPH is a hydrophilic azo initiator, it initially generates free peroxyl radicals in the aqueous region that gain access to and modify amino acid residues in the extramembranous regions of proteins. However, the peroxyl radicals produced can also attack phosphoglyceride esters in biological membranes and induce lipid peroxidation [42]. The decreased V_{max} indicates that some of the Na^+/Ca^{2+} exchangers are inactivated, and the structural changes detected by Western blot analysis corroborate this by showing fragmentation of a portion of the exchanger proteins (Fig. 5B). Additionally, aggregation of the proteins that was observed following exposure to AAPH could also be contributing to the decrease in maximal transport capacity.

Peroxynitrite is a highly reactive oxidant that is thought to be associated with several pathological states in neurons [34,43,44]. This agent readily permeates cell membranes [35] and can oxidize sulfhydryls, unsaturated fatty acids, and several organic molecules [43,45,46]. It can also form nitrotyrosine by nitrating the *ortho* position of tyrosine residues on specific proteins [47,48]. Exposure of SPMs and transfected CHO-K1 cells expressing the NCX1 to peroxynitrite resulted in a decrease in the ^{45}Ca uptake activity (Figs. 2 and 4A), an increase in the K_{act} for Ca^{2+} and no change in the V_{max} (Fig. 4B). Since both AAPH and peroxynitrite produced aggregation but only peroxynitrite decreased affinity for Ca^{2+}, the interaction of peroxynitrite with the protein seems to be selective. The increase in K_{act} for Ca^{2+} may result from a peroxynitrite-induced modification of a specific amino acid such as cysteine or tyrosine near the Ca^{2+} binding site, since peroxynitrite is likely to react with only a limited number of residues. Once all of the available sites have been modified, addition of higher concentrations of peroxynitrite is not likely to produce further alterations. The fact that a maximal inhibition of only 40–50% of the exchanger activity was observed is consistent with the presence of a limited number of reactive sites in the NCX protein. Once all such target residues are oxidized, addition of more peroxynitrite would not be expected to produce a greater effect. Although the amino acids that constitute the Ca^{2+} binding site in the exchanger protein are not yet known, these results suggest that amino acids with which peroxynitrite reacts participate in that binding site. The twofold increase in the K_m for Ca^{2+} is consistent with this interpretation.

It is encouraging that our results showing a decrease in the Na^+/Ca^{2+} exchanger after in vitro exposure to ROS are similar to those reported by other investigators using a variety of free radical generating systems. Dixon et al. [49] demonstrated a decrease in the Na^+-dependent ^{45}Ca uptake after the exposure of rat cardiac sarcolemmal vesicles to superoxide ion radicals and hydroxyl radicals. Inhibition of the electrogenic Na^+/Ca^{2+} exchange current has been shown for both photoactivated rose bengal and hypoxanthine plus xanthine oxidase in intact cardiac myocytes [17]. In contrast, other studies in ventricular myocytes have reported that ROS can stimulate Na^+/Ca^{2+} exchanger activity [50]. Studies in isolated sarcolemmal vesicles have yielded conflicting results, with some results showing stimulation and others showing inhibition of exchanger activity by ROS [15,49]. Our studies are the first to demonstrate structural alterations in the exchanger protein as a result of exposure to oxidative stress. The nature of the sites within the protein that are altered by interactions with ROS likely determine the ultimate effect on its ion transporting activity, and the interaction between ROS and the given protein may be fairly selective. For example, Viner et al. [51] demonstrated that nitrotyrosine formation is selective to one isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). The in vitro exposure of skeletal muscle sarcoplasmic reticulum vesicles of young rats to peroxynitrite yielded selective nitration of the SERCA2a isoform, even in the presence of an excess of SERCA1a. Similarly, the interaction of peroxynitrite with NCX may be targeted to a region where Ca^{2+} binds to the protein.

Our studies do show that the Na^+/Ca^{2+} exchanger is a possible target for ROS. If the aging process in brain is associated with enhanced levels of ROS, the exchanger activity might be altered. The increase in K_{act} for Ca^{2+} in the NCX1 isoform after in vitro exposure to peroxynitrite does parallel the effects we previously observed in the exchanger activity in aged SPMs [27]. Kinetic characterization of the Na^+-dependent Ca^{2+} transport in SPMs from brains of adult (5–7-month-old) and aged (23–25-month-old) rats revealed that the Ca^{2+} affinity of the transport system is decreased by a factor of 1.3 with no significant change in maximal transport capacity. Although it is not possible at this point to claim that oxidative modifications underlie the age-related changes, it is of interest to note that this biologically relevant oxidizing species had an effect on the kinetic properties of the Na^+/Ca^{2+} exchanger similar to that observed in aging brain. The NCX1, as well as other isoforms of the Na^+/Ca^{2+} exchanger, could be a target for ROS, resulting in the decrease of the exchanger transport function and, ultimately, in the disruption of the Ca^{2+} homeostasis associated with aging. High level expression of individual isoforms of the exchanger protein should make it possible to determine which regions of the protein are susceptible to modifications by specific ROS and how the transport activity in given tissues can be inhibited under conditions of oxidative stress.

Acknowledgements

This work was supported by NIH grants AG12993 and AA04732. We thank Dr. Nadia Gabellini, University of Padua, Italy, for providing us with the plasmid pcNCE6.0 of the canine heart NCX1, and Drs. Christian Schöneich and Andreas Hühmer, University of Kansas, for providing us with AAPH and peroxynitrite. Special thanks to Dr. Schöneich for providing helpful suggestions on the manuscript.

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