Na-K-ATPase in rat cerebellar granule cells is redox sensitive

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EFFICIENT FUNCTION OF THE Na-K-ATPase is crucial for neuronal survival. Treatment of cortical neurons in culture with the selective inhibitor of the Na-K-ATPase ouabain induced concurrent neuronal apoptosis and necrosis. Suppression of the Na-K-ATPase has been reported under pathological conditions associated with development of oxidative stress such as Parkinson’s and Alzheimer’s disease, ischemia-reperfusion injury, apoptosis, intoxication, and aging (6). Application of antioxidants and maneuvers efficiently reducing free radical production has been shown to restore Na-K-ATPase activity and rescue cell survival (7, 15, 16, 45). Although oxidative damage of the Na-K-ATPase is broadly reported, mechanisms of redox-induced regulation of the enzyme are not fully understood. Decreases in the amount of reduced thiols groups have been shown after exposure of the ATPase to millimolar H2O2 concentrations (4, 9). However, in intact cells, such a high concentration of the oxidant is rarely achieved. Instead, it is postulated that increased free radical production gives rise to local shifts in redox state. The impact of other factors, such as shifts in ATP levels, Na-K-ATPase phosphorylation, glutathionylation, or nitrosylation in response to oxidative stress, remain to be elucidated (4, 45). Interestingly, not only accumulation of oxidants but also abnormally elevated levels of reductants such as homocysteine provoke a memory deficit (38) and result in inhibition of Na-K-ATPase in hippocampus (39, 40, 50). Symptoms reported for hyperhomocysteinemia are characteristic for oxidative stress (41, 47). Thus the question arises as whether shifts in redox state occurring under physiological and pathological conditions are involved in the Na-K-ATPase regulation and, if so, what are the mechanisms involved in the redox-induced regulation of the Na-K-ATPase?

To address these questions, we made use of dispersed rat cerebellar granule cells. Neonatal rat cerebellum is rich in neurons (30). Cerebellar granule cells are easy to disperse without damaging cell membranes up to the age of postnatal day (P) 15. The Na-K-ATPase activity in cerebellar homogenate is as high as in cortex, making these cells an appropriate model for studying regulation of this ion transport system (42, 43). Thiol redox state of cerebellar granule cells is easily manipulated by using conjugating agents and permeable reductants. The unusually low exchange rate of reduced glutathione (GSH) between the cytosolic and mitochondrial pools [half-time (1/2) ~ 70 h] reported for neurons suggests that GSH content in these pools may be selectively altered also in isolated granule cells (11). The latter was achieved by exposing cerebellar granular cells to a blocker of GSH synthesis, butionine sulfoximine (BSO), for 12 h. A fivefold decrease in cytosolic GSH content in BSO-treated cells was not followed by shifts in mitochondrial GSH concentration (49). In our study, we used two conjugating agents, one of which, chloro- dinitrobenzene (CDNB), is known to penetrate both plasma and mitochondrial membranes, whereas permeability of the other, diethyl maleate (DEM), through the mitochondrial membranes is limited (10, 17, 26). Cell loading with GSH was achieved by treatment with permeable ethyl ester of GSH that is transferred to GSH by cytoplasmic esterases. Our data reveal that Na-K-ATPase function is sustained at maximal levels within an optimal redox potentials window. Both loading of cells with GSH and depletion of cytosolic and mitochondrial GSH pools results in suppression of transport activity of the Na-K-ATPase. Activity of the enzyme is controlled by the redox-induced shifts in free radical production rate and, when the mitochondrial pool of GSH is depleted, by cellular ATP depletion.

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MATERIALS AND METHODS

Isolation of dispersed rat cerebellar granule cells and cellular GSH manipulation. Rat pups were obtained from Wistar females kept on a standard diet in sterile facilities of the Vetsuisse Faculty of the University of Zürich in accordance with the animal handling guidance approved by the Veterinäramt des Kantons Zürich. Rat pups of P9–P13 were anesthetized with halothane and decapitated. Cerebellum was removed and digested with collagenase (final sp act 200 U/ml; Wako, Japan, or Type 4 from Warthington; see Refs. 8 and 43). Cell suspensions containing 1–2 × 10⁶ cells/ml were prepared on Tyrode solution containing (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES-NaOH, pH 7.4, at 37°C supplemented with 0.25% vol/vol FBS, passed through a 40-µm Falcon nylon filter, and used for experiments after 30-min restitutition time. Characterization of cells in suspension was done using anti-unique β-tubulin (TUJ-1) mouse monoclonal (BabCO; Covance) and anti-glia fibrillary acidic protein (GFAP) mouse monoclonal (Sigma) antibodies as markers for neurons and astrocytes, respectively. Details of the staining procedure are described elsewhere (36). Briefly, three aliquots of fresh isolated cerebellar granule cell suspension were washed in PBS with 2% FCS and 0.1% sodium azide, fixed with 2% paraformaldehyde for 20 min on ice, washed, and incubated in PBS with 0.5% saponin (PBS-saponin) for 20 min. After triple washing in PBS-saponin, two aliquots were incubated for 30 min on ice with either anti-TUJ-1 (1:5,000) or anti-GFAP (1:5,000) primary antibodies diluted on PBS-saponin. After triple washing, all three aliquots were incubated for another 30 min with Alexa Fluor goat anti-mouse secondary antibody (1:500; Molecular Probes) for 30 min, washed, and finally stained with 30 nmol 4′,6-diamidino-2-phenylindole dilactate for 1–2 min on ice. The number of TUJ-1- and GFAP-positive cells was counted using an Axiovert 200M fluorescent microscope (Zeiss) and normalized to total cell number.

In some experiments, the microsomal fraction isolated, as described earlier (23), from cerebral gray matter of adult Wistar rats was used.

Flow cytometry was used to monitor changes in nonprotein thiol levels and free radical production in the intact neuronal population. The latter was chosen according to cell size (forward scatter) and granularity (side scatter), as described in detail elsewhere (8, 43). Cells with damaged membranes were detected using propidium iodide (PI). PI was added to the cell suspension at final concentration of 1 μg/ml 1–2 min before measurements. The number of PI-positive cells in a preselected neuronal population was used to assess cell mortality. Thiol levels and free radical production in the selected neuronal population were assayed in PI-negative cells. Wherever experiments were done on crude suspensions of dispersed cerebellar granule cells, cell viability was tested by counting trypan blue-positive cells in a Burker chamber.

To deplete cellular GSH, CDNB and DEM were used from 100-mM stocks prepared on DMSO. A 500-mM stock solution of monooethyl ester of reduced glutathione (et-GSH) in DMSO was used to load the cells with GSH. Stock solution of glutathione synthetase inhibitor, BSO of 100 mM, was prepared in distilled water. As a rule, cells were preincubated at 37°C with 1 mM CDNB or 5 mM et-GSH for 30 min and with 1 mM DEM for 1 h. GSH (50 mM solution) was prepared in distilled water and buffered with Tris to the pH of 7.4. Both et-GSH and GSH stocks were freshly prepared before each experiment. When not stated otherwise, chemicals were purchased from Sigma (St. Louis, MO), and fluorescent dyes were obtained from Molecular Probes.

Determination of intracellular GSH, GSSG, and ATP. Quantification of total nonprotein reduced thiol levels, of which GSH is the most abundant, was performed spectrophotometrically. After incubation with or without conjugating agents or et-GSH, cells were lysed in 5% TCA, and proteins were removed by centrifugation. External et-GSH was removed by triple washing before cells were lysed. Reduced thiol levels were evaluated in supernatants using Ellmann’s reagent [5,5′-dithiobis(2-nitrobenzoic acid)]. Optical density of the resultant colored thionitrobenzoate anion was determined at 412 nm using a Lambda 25 spectrophotometer (Perkin-Elmer). Details of the analytical protocol are described elsewhere (44).

For GSSG determination, samples were analyzed in duplicates. One sample was preincubated for 20 min with 50 μM β-NADH; 3 and 0.1 mg/ml glutathione reductase (both purchased from Sigma) to reduce oxidized glutathione GSSG to GSH. The other one remained untreated and was used for cellular GSH measurements. GSH levels obtained for the pretreated samples gave total (GSSG + GSH) levels from which GSSG concentration was calculated by subtraction of reduced cellular GSH values from total and dividing of the obtained difference by two. In addition, relative changes in reduced nonprotein thiol levels in living neurons were evaluated by means of flow cytometry using chloromethylfluorescein (CMF)-diacetate (DA). After incubation with or without conjugating agents, cells were washed and loaded with CMF-DA (30 μM for 30 min). Nonfluorescent CMF-DA penetrates the cell membrane and is converted to CMF by esterases when in the cytosol. CMF forms conjugates with nonprotein reduced thiols in a reaction catalyzed by glutathione transferase. Specificity of conjugation reaction has been reported elsewhere (18). Fluorescent signal was monitored (λex 492 nm and λem 516 nm). Relative changes in fluorescence triggered by GSH depletion were later normalized to the signals in corresponding time-matched non-treated controls.

Cellular ATP was measured in protein-free cell lysates prepared by mixing cell suspension with equal amounts of 5% trichloroacetic acid using an ATP bioluminescent assay kit (Sigma), and luminescence was detected with Sirius luminoimeter (Bertold Detection Systems).

Measurements of hydrolytic and transport activities of Na-K-ATPase. Hydrolytic activity of the Na-K-ATPase was quantified as ouabain-sensitive inorganic phosphate production in cell homogenates or microsomal preparations. Intact cells or microsomes were incubated at 37°C with or without et-GSH, DEM, or CDNB. Thereafter, cells were destroyed by repeated freezing-thawing cycles, and cell homogenates or microsomes were preincubated with or without inhibitor of the Na-K-ATPase ouabain (1 mM final concentration) for 5–10 min in the media containing (in mM) 130 NaCl, 20 KCl, and 3 MgCl₂. Na-K-ATPase-induced ATP hydrolysis was measured as a difference in ATP cleavage rate in the presence or absence of inhibitor. ATP concentration used was either constant (3 mM) or variable in the range of 50 μmol-3 mM when affinity of Na-K-ATPase to ATP has been studied. Method developed by Rathbun and Betfach (32) was used to quantify the amount of inorganic phosphate spectrophotometrically.

Transport activity of the Na-K-ATPase was evaluated in intact cells using ⁸⁶Rb as a radioactive tracer for K⁺. Ouabain-sensitive unidirectional K⁺(⁸⁶Rb) influx was measured in cell suspensions prepared with 0.25% vol/vol PBS-containing Tyrode solution. Cells were preincubated at 37°C for 1 h with or without 1 mM DEM or for 30 min with or without 5 mM et-GSH or 1 mM CDNB. To distinguish between active, Na-K pump-mediated, and passive K⁺ influx, ouabain at the final concentration of 1 mM was added to one-half of the samples 15 min before the addition of the radioactive tracer. Flux measurements were started by adding ⁸⁶RbCl (~0.5 μCi/ml cell suspension; Perkin-Elmer). Aliquots of suspension of 0.9 ml were collected after 3, 5, and 10 min of incubation with ⁸⁶Rb, and flux was stopped by immediate dilution with 10 ml ice-cold washing medium [100 mM Mg(NO₃)₂ and 10 mM imidazole, pH 7.4, at 4°C]. After additional washing from external ⁸⁶Rb, cells were lysed in 5% TCA. Radioactivity of cells (A₀) and incubation medium (Aᵢ) were measured using a Tri-Carb 1600 TR liquid scintillation counter (Packard) in water phase (Cherenkov effect). Accumulation of ⁸⁶Rb in neurons was linear within 10 min incubation time with the tracer. Unidirectional fluxes (J) were calculated using the following equation:
where $A_n$ and $A_m$ are radioactivity of cells in 1 ml suspension and 1 ml medium, respectively; $m$ is amount of protein (mg/ml cell suspension) corrected for the amount of viable cells in suspension, $[K^+]_i$ is $K^+$ concentration in the incubation medium, and $r$ is the equilibration time with the tracer.

**Evaluation of mitochondrial vs. cytosolic ROS production.** Two fluorescent dyes were used to evaluate ROS production in neuronal suspension [2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and dihydorhodamine 123 (DHR 123)]. Both dyes are oxidized by peroxynitryl anion (ONOO$^-$) and hydroxyl radical (OH$^-$) directly, as well as by H$_2$O$_2$ in the presence of peroxidases, cytochrome c, or Fe$^{2+}$-containing enzymes (24, 34). Distribution of reduced and oxidized forms of these dyes differs substantially. Noncharged nonfluorescent H$_2$DCF upon cleavage by cytosolic esterases. The latter is retained in the cytosol and gets oxidized solely by cytosolic ROS, forming fluorescent DCF (λex 485 nm and λem 525 nm). However, one should consider that exchange of cytosolic and mitochondrial pools of H$_2$O$_2$ may occur. DHR is a noncharged nonfluorescent membrane-permeable molecule. The reduced form of the dye enters both cytosolic and mitochondrial compartments, whereas the positively charged oxidized form (rhodamine 123, λex 505 nm and λem 525 nm) preferably accumulates in mitochondria. Therefore, when measured under similar conditions, fluorescent responses of two dyes reflect acute changes in ROS levels only in cytosol or both in mitochondria and cytosol, respectively.

**Stock solutions of both dyes were prepared with DMSO.** Cells were loaded for 30 min with H$_2$DCFDA or DHR 123 at a final concentration of 100 μM and 10 μM, respectively. DMSO levels in cell suspension during loading did not exceed 0.1%. When loading was complete, the cells were washed free of extracellular dyes and resuspended in FBS-containing Tyrode solution and treated with conjugating agents or et-GSH. Changes in fluorescence were normalized to nontreated control cells.

**Evaluation of peroxidase activity.** Peroxidase activity was measured by an adaptation of the method described by Mullane et al. (27). After 30 min incubation with or without 1 mM CDNB, cells were centrifuged by a single centrifugation (3 min at 500 g), resuspended in phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (HETAB), and destroyed by several freezing-thawing cycles in liquid nitrogen. Membrane debris was removed by a brief centrifugation at 16,000 g. Peroxidase activity in supernatant was assayed in duplicate by mixing 50 μl supernatant with 50 μl of 0.025% O-dianisidine hydrochloride. Reactions were started by adding 50 μl of 0.01% H$_2$O$_2$. After 3 min of incubation at room temperature, the reaction was stopped by adding 10 μl of 1% Na$_2$S$_2$O$_3$ solution. For each sample, negative control was made where H$_2$O$_2$ was added after the reaction was stopped. Values obtained were then subtracted from the corresponding sample readouts. Changes in optical density were measured at 460 nm using the ELISA plate reader (Multi-skran RC; ThermoLabystems). Calibration was done in duplicate with myeloperoxidase dissolved in phosphate buffer with 0.5% HETAB to the activity of 0, 2, 4, 8, 15, 30, and 50 μlU. Activity detection limit was set to be ≥2 μlU. Activity was normalized to the amount of protein in cell homogenate.

**Statistical approaches.** Data were analyzed using GraphPad Instat software and presented as means ± SE. The following symbols were used in Figs. 1–6 to express statistical differences between two groups of data: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

**RESULTS**

**Characterization of subpopulations in dispersed cerebellar granule cell suspension.** The population of dispersed cerebellar granule cells consists mainly of neurons but is not homogenous (8, 30, 43). Cell selection by size and granularity performed by means of flow cytometry allows exclusive operation with the neuronal subpopulation (8, 43). Figure 1 exemplifies gating parameters that we have used to select for a viable neuronal cell subpopulation. The cells identified as neurons in our experiments are located within the R1 frame (Fig. 1A). Within this population, cells with damaged membranes were revealed by PI accumulation (excitation wavelength λex 488 nm and emission wavelength λem 617 nm). These cells are located in the frame R3. Their amount never exceeded 10% at the beginning of experiments. Only the homogeneous PI-negative population of neuronal cells (frame R2 in Fig. 1B) was used for further analysis. Considering the fact that the selection procedure affects viability of granule cells, we did not acquire it for the suspensions used in experiments where transport and hydrolytic activity of the Na-K-ATPase, GSH/GSSG, and ATP levels were monitored in cells treated with et-GSH or conjugating agents. To characterize the number of neurons and astrocytes in crude dissociated cerebellar cell suspension, cells were fixed with paraformaldehyde, permeabilized with saponin, and stained with neuronal and astrocyte markers (TUJ-1 and GFAP, respectively). Cell counting revealed that ~20% of total cell number was GFAP positive, ~70% was TUJ-1 positive, and the rest represented cells of nonneuronal and nonastrocytic origin.

**Manipulation of cellular GSH levels by DEM, CDNB, and et-GSH.** Cellular GSH levels were modified using the conjugating agents DEM and CDNB, and et-GSH. Monitoring of cellular GSH content in the selected neuronal population and in crude suspension of cerebellar granule cells was performed using flow cytometry and Ellmann’s calorimetric assay, respectively. In the sorted neuronal population and in the crude cerebellar granule cell suspension, DEM was less efficient in GSH depletion than CDNB (Fig. 2A and B). GSH depletion was completed at 60 min in DEM-treated cells and at 15–20 min in cells exposed to CDNB. Steady-state cellular GSH levels reached in cells exposed to DEM were higher compared with CDNB-exposed cells, as determined by Ellmann’s reagent in crude suspension (Fig. 2A) and using fluorescent dye in selected neuronal populations (Fig. 2B). In the latter, minimal
Methylfluorescein diacetate. Values are means of 3–4 independent experiments with CDNB using fluorescent label for nonprotein reduced thiol chloroformate, and CDNB using fluorescent label for nonprotein reduced thiol chloroformate. Values are means of 5 independent experiments without CDNB, DEM, and et-GSH from the corresponding time-matched controls. Exposure of granule cells to et-GSH for 30 min resulted in a substantial increase in intracellular nonprotein reduced thiol levels (Fig. 2A). Notably, GSSG concentrations in CDNB-treated cells did not differ from those in control during 30 min of incubation with CDNB (data not shown).

Moreover, we have used an inhibitor of glutathione synthase, BSO, to validate the potential impact of de novo GSH synthesis in maintenance of GSH concentration in GSH-depleted granule cells. Treatment with 100 µM BSO did not substantially affect GSH levels within 1 h in either control cells or the ones after GSH depletion, thereby assuring stability of GSH levels during the experimental run making up 34.3 ± 3.5 and 12.5 ± 1.3 nmol/mg protein for control and CDNB-treated cells after 30 min of incubation, respectively.

Treatment of crude suspension of cerebellar granule cells with 5 mM et-GSH for 30 min resulted in a substantial increase in intracellular nonprotein reduced thiol levels (Fig. 2A).

Viability of cells with modified redox state. Changes in viability of cerebellar granule cells in response to modification of cellular GSH levels were assayed by monitoring the amount of PI-positive cells for selected neuronal cell populations using flow cytometry and as trypan blue-positive cells in nonselected dispersed granule cells.

In a selected neuronal population, the amount of PI-positive cells at the beginning of experiments did not exceed 10%. After 30 min of treatment with CDNB, the number of neurons with damaged plasma membranes increased significantly compared with that in untreated cells (Fig. 2C). Incubation of cells with et-GSH for 30 min also caused a modest decrease in neuronal viability compared with the corresponding time-matched controls (Fig. 2C). Survival of CDNB-treated neurons was rescued by addition of et-GSH. The amount of PI-positive cells after 1 h exposure to DEM did not differ from that in nontreated controls.

The amount of trypan blue-positive cells in crude suspension was always <50% of the total cell number. In contrast to that for a selected neuronal population, incubation of crude granule cell suspension with DEM for 60 min caused an increase in mortality compared with time-matched controls. Treatment with CDNB also resulted in granule cell mortality comparable to that monitored by flow cytometry for a selected neuronal population (31 ± 4% increase in trypan blue-positive cells compared with nontreated time-matched controls). Exposure of crude cerebellar granule cell suspension to et-GSH for 30 min did not affect viability significantly. Notably, crude suspension contained 70% neurons, 20% astrocytes, and 10% of other cells.

Effect of GSH depletion and GSH overloading on hydrolytic and transport activities of the Na-K-ATPase. The first set of experiments was designed to test for possible direct action of both conjugating agents, as well as of et-GSH on the Na-K-ATPase hydrolytic function in microsomes prepared from rat cerebral cortex. In the microsomal fraction, neither CDNB, DEM, nor et-GSH affected the Na-K-ATPase-mediated ATP cleavage rate of 158 ± 21 µmol Pi/mg protein h⁻¹. Thus these reagents did not affect the membrane-bounded enzyme directly. Furthermore, hydrolytic activity of Na-K pump was measured after alteration of cellular GSH content of intact granule cells in crude suspension (Fig. 3). Exposure of granule cells to DEM and et-GSH did not
affect hydrolytic activity of the Na-K-ATPase, whereas treatment with CDNB stimulated it. Moreover, evaluation of the Na-K-ATPase affinity to ATP revealed higher affinity in CDNB-treated cells with apparent $K_m$ shifting from $0.39 \pm 0.04$ to $0.21 \pm 0.03$ mM. Because CDNB did not affect the enzyme activity in the microsomal fraction, the existence of some cytosolic factors involved in CDNB-induced regulation of the Na-K-ATPase function can be suggested.

Transport activity of the Na-K pump in crude cerebellar granule cell suspensions treated with conjugating agents or et-GSH was evaluated as ouabain-sensitive $K^{+}$ influx ($^{86}$Rb$^+$). As shown in Fig. 4A, 30 min exposure to 5 mM et-GSH caused substantial inhibition of active $K^+$ influx. To elucidate whether extracellular or intracellular GSH suppresses Na-K pump transport function, active $K^+$ influx was further measured in cells pretreated with 5 mM impermeable GSH or loaded with 5 mM et-GSH. Upon loading, cells were washed from the extracellular reducing agent before addition of the radioactive tracer. Figure 4B shows inhibition of the transport activity of the Na-K pump in response to an increase in either solely extracellular or only intracellular GSH. Elevation of both extracellular and intracellular GSH was followed by even more potent inhibition of active $K^+$ influx compared with that caused by extracellular GSH only (Fig. 4B). The latter observation implies existence of two independent mechanisms of the Na-K pump inhibition by extracellular and intracellular GSH.

Whereas treatment with DEM had no effect on the Na-K pump function (Fig. 4A, inset), a marked suppression of ouabain-sensitive $K^+$ influx was observed in CDNB-treated cerebellar granule cells (Fig. 4A). This result did not correlate with the data obtained for hydrolytic activity of the Na-K-ATPase measured in cells exposed to CDNB, suggesting that substrate availability rather than changes in enzyme functional properties in response to GSH depletion was a factor limiting transport activity of the pump.

ATP levels in GSH-depleted and GSH-loaded neurons. Indeed, as shown in Fig. 5B, GSH deprivation caused by CDNB treatment was followed by ATP depletion that was significant already after 10 min of exposure to the conjugating agent. Although the decrease in GSH concentration was rapid ($t_1/2$ 2–3 min; see Fig. 5B, inset), ATP levels showed modest reduction after 10 min of incubation, with the latter rapid depletion phase between 10 and 30 min of incubation. Exposure to DEM or et-GSH was without an effect on cellular ATP levels (Fig. 5A). Notably, changes in hydrolytic activity of the Na-K-ATPase were observed after incubation of the cells with CDNB for 10–15 min or more, suggesting that the enzyme responded to the changes in ATP levels triggered by GSH depletion (Fig. 5B).
Because CDNB is reported to penetrate the mitochondrial membrane and efficiently deplete the mitochondrial GSH pool in contrast to DEM (10), the following ATP depletion may result from mitochondrial oxidative damage. Thus we proceeded with monitoring of free radical production in cerebellar granule cells treated with CDNB, DEM, and et-GSH.

**ROS levels in cytosol and mitochondria of cells treated with CDNB, DEM, or et-GSH.** To visualize free radical production, we used two fluorescent dyes targeting radicals either presumably in the cytosol (H$_2$-DCFDA) or in both cytosolic and mitochondrial (DHR 123) compartments. As shown in Fig. 6, A and B, incubation of granule cells with 1 mM DEM for 60 min did not result in changes in fluorescence of both dyes used. The level of intracellular fluorescence measured after incubation of cells with CDNB depended strongly on the dye used. Fluorescence intensity of DCF decreased compared with non-

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**Fig. 5. ATP levels in cerebellar granule cells after GSH depletion or loading.**

A: ATP levels were measured after 30 min treatment of cells with or without 1 mM CDNB or 5 mM et-GSH for 30 min or 1 mM DEM for 60 min. Data are means of 5 independent experiments ± SE. **P < 0.01. B: kinetics of GSH depletion (inset) and corresponding decrease in intracellular ATP in crude suspension of granule cells exposed to 1 mM CDNB (●) and control cells (○). Data are from 5 independent experiments ± SE.

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**Fig. 6. Free radical production as a function of thiol redox state in selected neuronal subpopulation of cerebellar granule cells.**

A: fluorescence intensity of DCF was recorded for cells pretreated with 1 mM DEM (60 min), CDNB, or 5 mM et-GSH (30 min) and normalized to nontreated control cells. Data are means of 3–7 independent experiments ± SE. **P < 0.01. B: recordings of rhodamine 123 fluorescence intensity in neuronal subpopulation. Data are means of 3 or 4 independent experiments ± SE. C: intracellular GSH accumulation (●) and free radical production monitored as DCF accumulation (○) as a function of et-GSH concentration in the incubation medium. Each curve represents average values of 3 independent experiments ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001.
treated control (see Fig. 6, A and B), whereas that of DHR-treated neuronal population cannot be attributed to quenching resulting from CDNB treatment. Because oxidation of HOCl by H2O2 only occurs when catalyzed by peroxidases or in the presence of cytochrome c or Fe2+-containing enzymes (the latter are abundant in mitochondria but not in the cytosol), one could imagine that activity of cytosolic peroxidases was suppressed in GSH-depleted cells. In nontreated cerebellar granule cells, peroxidase activity made up ~15 mU/mg protein, whereas in CDNB-treated cells, it was below detection limits of 2 mU/mg protein.

The signal obtained from DHR was higher in CDNB-treated cells compared with the untreated control. Similar to H2-DCF, H2O2-induced oxidation of DHR only occurs when catalyzed by peroxidases, cytochrome c, or Fe2+-containing enzymes as well as by ONOO− (24, 34). However, in contrast to H2-DCF, DHR rapidly penetrates mitochondrial membranes where numerous mitochondrial Fe2+-containing proteins may catalyze oxidation of DHR by H2O2, even in the absence of functional peroxidases. Thus the conclusion can be made that upregulation of free radical production in CDNB-treated cells occurred primarily in mitochondria.

Treatment with et-GSH was followed by a pronounced increase in the fluorescent signal of both dyes. Notably, stimulation of free radical production could only be seen when cellular GSH concentrations increased significantly (Fig. 6C). Incubation of cells with 5 mM of impermeable GSH did not result in upregulation of ROS production (data not shown).

**DISCUSSION**

GSH concentrations in mammalian brain vary in the range of 1 to 3 mM, depending on the regions of the brain and age of the animals (12). GSH levels in developing rat cerebellum have been shown to remain constant from P3 to P9 but increased sharply between day 9 and day 11 from 1.57 ± 0.06 to 2.04 ± 0.06 μmol/g wet wt, as measured by liquid chromatography (33). In our hands, GSH concentrations of ~30 nmol/mg protein were stable for the P9-P10 animals, with significant deviation observed from at P11 and older. Cellular GSH concentration was identical to that reported for cerebellar granule cell suspensions isolated from P8 rats and cultured ex vivo (37). GSH levels detected chromatographically were somewhat lower than those measured photometrically using Elamnn’s reagent because of the inability of photometric detection technique to discriminate between GSH and other nonprotein thiols.

GSH is a major determinant of the cellular redox state protecting SH-groups of proteins from oxidation and functioning as an electron donor in numerous redox reactions, including those in which glutathione peroxidase is involved. Using conjugating agents and et-GSH, we were able to deplete cells of GSH, with maximal depletion after 30 min treatment with CDNB or an increase in cellular GSH concentrations to ~0% and more. Steady-state GSH concentrations reached after CDNB (30 min) and DEM (1 h) treatment corresponded to 13 and 65% GSH depletion in a selected neuronal population (Fig. 2B) and to 35 and 47% in crude granule cell suspensions (Fig. 2A). On the basis of earlier reports on the efficiency of DEM and CDNB in depleting mitochondrial and cytosolic GSH and our observations, we suggest that the mitochondrial GSH pool remained intact in DEM-treated cells but was at least partially depleted in cells exposed to CDNB (17, 26). Redox couple GSSG/GSH is accepted as the most reliable indicator of the cellular redox state. Therefore, an equation describing the GSH half-cell reduction potential Ehc can be used to estimate the changes in cellular redox potential upon GSH depletion and loading (35).

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E_{hc} (mV) = -240 - (59.1/2) 
\times \log ([GSH]^2/[GSSG]) \text{ at } 25^\circ C, \text{ pH 7.0}
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Treatment with CDNB resulted in a shift in redox potential from −336 to −254 mV (ΔE = 82 mV), whereas incubation with 5 mM et-GSH caused a modest reduction to approximately −370 mV (ΔE = −34 mV). As follows from our observations (Fig. 4), shifts in redox potential levels in both directions result in inhibition of the Na-K-ATPase transport activity.

Depletion of cellular GSH over a threshold of ~30% induced by CDNB treatment was followed by a progressive decrease in viability of cerebellar granule cells. Toxicity of CDNB could be detected in both the neuronal fraction as a number of PI-positive cells and in the crude granule cell suspension as a trypan blue-positive cell fraction. DEM did not cause increased mortality in the selected neuronal population but gave rise to a decrease in viability in the crude cell suspension. As we have shown, the crude granule cell suspension contains ~30% of cells of nonneuronal origin (astrocytes, pericytes, and fibroblasts). Therefore, discrepancies may be caused by differential sensitivity of these nonneuronal cells to cytosolic GSH depletion. Treatment of astrocyte primary cultures for 4 h with 0.1 mM DEM (10 times lower than we were using) was reported to be almost as potent in GSH deprivation as 1 mM CDNB was in our hands (29). Interestingly, loading of cells with GSH also caused an increase in the number of PI-positive cells (Fig. 2C) but was not detectable by counting of trypan blue-positive cells. An overall increase in the mortality resulting from manipulation of GSH content occurred but never exceeded 10% over nontreated time-matched controls in selected neuronal population and 30% in crude suspension.

An increase in neuronal mortality in CDNB- and et-GSH-treated cells was followed by upregulation in free radical production measured using fluorescent dyes. To avoid artifacts when judging free radical formation, both chemical properties of the dyes themselves and the mechanisms of their oxidation, which is then recorded as an increase in fluorescence, have to be considered. Treatment of cells with CDNB was followed by upregulation of free radical production as measured by DHR but not by H2-DCF. In fact, oxidation of H2-DCF was slowed down by CDNB treatment. Structures of the reduced and oxidized forms of the dyes given on the Web page of Molecular Probes (http://www.molecularprobes.com) reveal that electroneutral DHR will penetrate both plasma and mitochondrial membranes and will respond to the changes in free radical production in both cytosolic and mitochondrial compartments. When oxidized by O2•−, OH•−, and ONOO− as well as by H2O2 in the presence of Fe2+, cytochrome c, or peroxidases, the dye will transfer to positively charged rhodamine and accumulate in mitochondria. H2-DCF is negatively charged and will therefore remain in the cytoplasm, responding to the changes in cytosolic free radical production and to H2O2.
leaking from mitochondria. Oxidation of H$_2$DCF by H$_2$O$_2$ also requires the presence of catalysts such as iron, cytochrome $c$, or peroxidases (24). In hepatocytes, known to contain iron deposits in perinuclear space (31), CDNB triggers an acute massive increase in fluorescent intensity (3). The fact that in the neuronal fraction of cerebellar granule cells, oxidation of the dye is slowed down in the presence of CDNB does not reflect changes in H$_2$O$_2$ levels in cytosol but most probably inhibition of peroxidases that in the absence of sufficient amounts of Fe$^{2+}$ are the only catalysts of H$_2$O$_2$-induced H$_2$DCF oxidation. No conclusion can be hence derived on the levels of H$_2$O$_2$ in the cytosol of CDNB-treated cells. An increase in DHR oxidation in response to CDNB application shows that free radical production is upregulated with no indication on the nature of free radicals produced (Fig. 6). Because peroxidases are inhibited by CDNB treatment, we suggest that the major sources of DHR oxidation are mitochondria, where cytochrome $c$ and other Fe$^{2+}$ proteins are present to catalyze H$_2$O$_2$-induced oxidation of DHR. An increase in free radical production in CDNB-treated mitochondria, as total GSH content was depleted by 40% or more, has been reported elsewhere (17). ATP depletion observed in CDNB-treated cells, in which GSH levels were making up 28% of nontreated controls (Fig. 5B), supports our suggestion on the mitochondrial origin of free radicals detected by DHR. Indeed, depletion of the mitochondrial GSH pool was reported to cause oxidation of complexes I, II, and IV (25) with the consequent inhibition of ATP production. Mitochondrial complex I in neurons may, in addition, be inhibited by nitric oxide and peroxynitrite (12).

One more source of ATP deprivation in cells treated with conjugating agents is active extrusion of complexes of GSH with conjugating compounds (2). However, no changes in ATP levels were observed in DEM-treated cerebellar granule cells. In addition, ATP concentration in erythrocytes and hepatocytes exposed to CDNB remained unaltered (3, 5, 28), whereas, in granule cells, ATP levels decreased fourfold after CDNB treatment.

A massive increase in both DHR and H$_2$DCF oxidation was observed in GSH-overloaded cerebellar neurons (Fig. 6). Reductive stress caused by GSH overload, although followed by an increase in mortality, did not result in ATP depletion. The latter suggests that the nature of free radicals or/and availability of protein thiols for oxidation differed in GSH-depleted and GSH-overloaded granule cells. The mechanism of oxidative stress triggered by GSH loading remained out of the scope of this report. However, the literature available on the oxidation observed in patients with homocysteinuria and in vitro modeling of the process of auto-oxidation of GSH postulate the following processes to occur in GSH-overloaded cells (19, 48):

$$\text{GSH} + \text{Cu}^{2+}/\text{Fe}^{2+} \rightarrow \text{Cu}^{2+}/\text{Fe}^{3+} + \text{GS} + \text{H}^+ \quad (3)$$

$$\text{Cu}^{2+}/\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Cu}^{2+}/\text{Fe}^{3+} + \text{O}_2^- \quad (4)$$

$$\text{GS} + \text{GSH} \rightarrow \text{GSSG}^- + \text{H}^+ \quad (5)$$

$$\text{GSSG}^- + \text{O}_2 \rightarrow \text{O}_2^- + \text{GSSG} \quad (6)$$

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (7)$$

As follows from our observations, both treatment of cerebellar granule cells with CDNB and et-GSH resulted in suppression of the active K$^+$ influx (Fig. 4). However, mechanisms of inhibitory action of GSH depletion and loading on the Na-K-ATPase were clearly different. Hydrolytic activity of the enzyme was stimulated by CDNB and not affected in GSH-loaded cells (Fig. 3). Stimulation of hydrolytic activity of the Na-K-ATPase along with suppression of the transport function in cells treated with CDNB may originate either from a decrease in cellular ATP levels or from modulations of the enzyme triggered by free radical production. An increase in affinity of the enzyme to ATP was characteristic of a shift in the ratio of E1 (Na$^+$ conformation)-to-E2 (K$^+$ conformation) toward E1 (4). Studies done on the purified Na-K-ATPase preparation reveal that affinity of the oxidized protein to ATP is higher and to K$^+$ lower than that of the native enzyme (46). However, maximal hydrolytic activity ($V_{\text{max}}$) of the oxidized Na-K-ATPase decreased, which is not the case in CDNB-treated cerebellar granule cells (Fig. 3). Despite an increase in the enzyme affinity to ATP, its transport function in CDNB-treated cerebellar granule cells was suppressed because of ATP deprivation. Studies on the purified membrane-bound protein revealed that a decrease from 2.5 to 0.75 mM ATP corresponding to ~25 and 7.5 nmol/mg protein, respectively (see Fig. 5), results in an ~1.8- to 1.9-fold lower hydrolytic activity of the Na-K-ATPase (14). This observation is in good agreement with our data obtained for the transport activity of the Na-K-ATPase in CDNB-treated cells (Fig. 4). Moreover, local ATP concentration in the premembrane pool may change even more dramatically than the bulk one (20). Similar twofold inhibition of active K$^+$ uptake has been reported for hepatocytes, since bulk cellular ATP concentration decreased to 40% of control (1).

The fact that an increase in GSH concentration had no effect on the cellular ATP levels while free radical production was upregulated (Figs. 5 and 6) implies that GSH-induced inhibition of transport function of the Na-K-ATPase is not linked to the limitation in substrate availability. Furthermore, effects of intracellular and extracellular GSH on the Na-K-ATPase are additive, suggesting the existence of distinct intracellular and extracellular targets for reducing agents on the Na-K-ATPase itself or regulatory proteins. Because loading of cells with GSH was followed by an increase in free radical production both in mitochondria and cytosol, suppression of the enzyme activity could result from partial oxidation or modification of the Na-K-ATPase molecule such as glutathionylation (4). However, hydrolytic activity of the enzyme in et-GSH-treated cells remained unchanged, suggesting that no oxidation of thiol groups occurred. Impermeable GSH did not trigger a free radical burst; hence, its inhibitory action on the transport activity of the Na-K-ATPase is most probably mediated via reduction of extracellularly facing S-S bonds. Three of these are present in the $\beta$-subunit of the Na-K-ATPase. Their reduction results in suppression of the enzyme transport activity (21, 22). Some of these dithiol bridges are more susceptible to reduction and may be targeted by extracellular GSH when present in excessive amounts.

In conclusion, our study reveals that function of the Na-K-ATPase in cerebellar granule cells is redox sensitive. Transport activity of the enzyme is maximal within a narrow range of redox potentials and is suppressed both under conditions of oxidative and reductive stress. GSH-modified cerebellar granule cells with modified GSH content may be used as a model to study neuronal responses to physiological and pathological conditions.
stimuli. For example, GSH depletion similar to that observed in CDNB-treated cells is observed in dopaminergic neurons in the cause of Parkinson’s disease (12, 13). There, dopamine plays a role of conjugating agent, and dopamine-GSH complex is secreted from neuronal cells with the following development of oxidative stress, ATP depletion, and final cell death. An increase in reducing equivalents may be observed both under physiological (decrease in oxygenation) and pathological (hyperhomocysteinemia) conditions (5, 40). Future studies will help characterize changes in the Na-K-ATPase structure and its possible chemical modification that occurs in response to oxidative and reductive stress.

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