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Exacerbated Responses to Oxidative Stress by an Na^+ Load in Isolated Nerve Terminals: the Role of ATP Depletion and Rise of $[Ca^{2+}]_i$

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We have explored the consequences of a $[Na^+]_i$ load and oxidative stress in isolated nerve terminals. The Na^+ load was achieved by veratridine (5–40 μM), which allows Na^+ entry via a voltage-operated Na^+ channel, and oxidative stress was induced by hydrogen peroxide (0.1–0.5 mM). Remarkably, neither the $[Na^+]_i$ load nor exposure to H_2O_2 had any major effect on $[Ca^{2+}]_i$, mitochondrial membrane potential ($\Delta\psi$m), or ATP level. However, the combination of an Na^+ load and oxidative stress caused ATP depletion, a collapse of $\Delta\psi$m, and a progressive deregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis. The decrease in the ATP level was unrelated to an increase in $[Ca^{2+}]_i$ and paralleled the rise in $[Na^+]_i$. The loss of $\Delta\psi$m was prevented in the absence of Ca^{2+} but unaltered in the presence of cyclosporin A. We conclude that the increased ATP consumption by the Na,K–ATPase that results from a modest $[Na^+]_i$ load places an additional demand on mitochondria metabolically compromised by an oxidative stress, which are unable to produce a sufficient amount of ATP to fuel the ATP-driven ion pumps. This results in a deregulation of $[Na^+]_i$ and $[Ca^{2+}]_i$, and as a result of the latter, collapse of $\Delta\psi$m. The vicious cycle generated in the combined presence of Na^+ load and oxidative stress could be an important factor in the neuronal injury produced by ischemia or excitotoxicity, in which the oxidative insult is superimposed on a disturbed Na^+ homeostasis.

Key words: oxidative stress; Na^+ load; mitochondrial membrane potential; ATP depletion; Na^+ deregulation; Ca^{2+} deregulation

Oxidative stress has been associated with neuronal death observed in a variety of neurodegenerative diseases and in ischemia (Schmidley, 1990; Phillis, 1994) (see also Beal, 1995). Hydrogen peroxide is a convenient means to model oxidative stress, because the insult is relatively mild compared with that induced by other reactive oxygen species (Zoccarato et al., 1995; Tretter and Adam-Vizi, 1996), thus enabling the resolution of early alterations in cellular functions. It has been suggested that excessive production of this oxidant contributes to the pathogenesis of Parkinson's disease (Schapira, 1994) and cellular damage occurring during reperfusion (Turrens et al., 1991; Hyslop et al., 1995).

The dysfunctions developing in nerve terminals during acute exposure to the oxidant include depolarization of the plasma membrane, a small increase in resting $[Ca^{2+}]_i$ (Tretter and Adam-Vizi, 1996), and a decrease in the ATP level and [ATP]/[ADP] ratio (Zoccarato et al., 1995; Tretter et al., 1997). Although these changes are modest, the oxidant applied at small concentrations (<1 mM) is able to induce delayed cytotoxicity (Whittemore et al., 1995; Desagher et al., 1996; Gardner et al., 1997; Hoyt et al., 1997).

It appears that it could also be acutely more harmful when the oxidative stress is combined with other burdens. An implication for this has been provided by a recent observation that the membrane potential of *in situ* mitochondria ($\Delta\psi$m) is maintained in the presence of H_2O_2, but when complex I or the F_0F_1-ATPase are also inhibited, themselves without effect on $\Delta\psi$m, mitochondrial membrane potential collapses (Chinopoulos et al., 1999). It has also been reported that H_2O_2 potentiates a decrease in $\Delta\psi$m induced by glutamate excitotoxicity (Scanlon and Reynolds, 1998).

Oxidative stress is a condition that *in vivo* often occurs concurrently with other disruptions. In this study we specifically examined the energy state, mitochondrial function, and ion homeostasis in nerve terminals during H_2O_2-induced oxidative stress superimposed on a disruption in Na^+ homeostasis. The importance of this question is indicated by the observations showing that Na^+ entry is a critical factor in the cellular injury produced by ischemia/reperfusion (Waxman et al., 1994; Weber and Taylor, 1994; Probert et al., 1997; Stys and Lopachin, 1998; Zhang and Lipton, 1999) (see also Urenjak and Obrenovitch, 1996). Furthermore, it has been reported that disruption in $[Na^+]_i$ homeostasis developing during ischemia is worsened during reperfusion (Rose et al., 1998; Taylor et al., 1999). The mechanism of the exacerbated deregulation of ions is poorly understood. Injury induced by reperfusion is generally thought to be associated with increased production of reactive oxygen species (Cao et al., 1988; Halliwell, 1992; Siesjö et al., 1995).

Glutamate excitotoxicity is another condition involving both increase in $[Na^+]_i$ and oxidative stress. Excessive stimulation of NMDA receptors leads to an increase in $[Na^+]_i$ (Kiedrowski et al., 1994a,b) and has also been demonstrated to result in an overproduction of reactive oxygen species (Coyle and Puttfarcken, 1993; Lafon-Cazal et al., 1993; Patel et al., 1996).

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Our study, which is the first to address directly the role of Na^+ load in the acute cellular responses to oxidative stress, might aid in understanding the factors and mechanisms contributing to cellular injury and death in response to an oxidative insult.

MATERIALS AND METHODS

Preparation of synaptosomes. Isolated nerve terminals (synaptosomes) were prepared from brain cortex of guinea pigs by a method detailed previously (Adam-Vizi and Ligeti, 1984). Synaptosomes obtained from an 0.8 M sucrose gradient were diluted with ice-cold distilled water to a concentration of 0.32 M and then centrifuged at 20,000 × *g* for 20 min. The pellet was suspended in 0.32 M sucrose (20 mg/ml protein) and kept on ice, and 50 μl aliquots, for further manipulation, were incubated in a standard medium (in mM: 140 NaCl; 3 KCl; 2 $MgCl_2$; 2 $CaCl_2$; 10 PIPES, pH 7.38, and 10 glucose) at 37°C.

Determination of Δψm. Membrane potential of *in situ* mitochondria was determined by 5,5′, 6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a fluorescence probe that accumulates in mitochondria and forms J-aggregates from monomers. It has been demonstrated that both the fluorescence of J-aggregate at 590 nm (Reers et al., 1991) and that of the monomer at 530 nm (DiLisa et al., 1995) reflect Δψm. Synaptosomes suspended in Ca^{2+}-free standard medium were loaded with JC-1 (30 μM) for 15 min at 37°C. After sedimentation and washing, synaptosomes were resuspended (8 mg/ml), and for fluorescence measurements, 50 μl aliquots were diluted in 2 ml of standard medium. Fluorescence intensity was determined at 37°C in a PTI (Monmouth Junction, NJ) Deltascan fluorescence spectrophotometer. We have previously shown (Chinopoulos et al., 1999) that H_2O_2 causes a nonspecific decrease in the signal at 595 nm that is unrelated to Δψm, however fluorescence at 535 nm reliably reflects changes in Δψm, therefore we have used only the emission from the monomer recorded at 535 nm in the present study.

Determination of $[Na^+]_i$. Synaptosomes were loaded with sodium-binding benzofuran isophthalate (SBFI; 10 μM) by incubation in a standard medium, in which sodium had been iso-osmotically replaced with sucrose and Pluronic acid (0.3%) was added for 60 min at 37°C. As described previously (Deri and Adam-Vizi, 1993) the use of Na^+-free medium enables the monitoring of the dye accumulation in synaptosomes during the loading period. After sedimentation and washing, the pellet was resuspended (8 mg/ml), and 50 μl aliquots were used in a cuvette containing 1.5 ml of standard medium. The fluorescence of intrasynaptosomally trapped SBFI was measured using 340/380 nm excitation and 510 nm emission wavelengths in a PTI Deltascan fluorescence spectrophotometer at 37°C. A calibration curve to quantify $[Na^+]_i$ in millimolar concentration was constructed in the presence of 3 μM gramicidin in a medium containing different concentrations of Na^+, as described previously (Deri and Adam-Vizi, 1993).

Determination of $[Ca^{2+}]_i$. Nerve terminals were loaded with fura-2 by incubation in the standard medium containing 8 μM fura-2 AM at 37°C (4 mg/ml) for 60 min. After sedimentation and washing synaptosomes were resuspended in the standard medium to give an 8 mg/ml protein concentration, and 50 μl aliquots in 2 ml medium were used for determination. Fluorescence intensity was measured in a PTI Deltascan fluorescence spectrophotometer using 340/380 nm excitation and 510 nm emission wavelengths. $[Ca^{2+}]_i$ was calculated using the ratio calibration approach described by Grynkiewicz et al. (1985).

ATP and ADP measurement. ATP and ADP levels were determined according to the luciferin–luciferase method as described by Kauppinen and Nicholls (1986) and detailed previously (Tretter et al., 1997). Bioluminescence was detected with an LKB (Turku, Finland) Luminometer 1251. Results are expressed as nanomoles of ATP per milligram of synaptosomal protein and as [ATP]/[ADP] ratio.

Materials. Standard laboratory chemicals were obtained from Sigma (St. Louis, MO). Fura-2 and SBFI were purchased from Calbiochem (San Diego, CA), JC-1 was obtained from Molecular Probes (Eugene, OR).

Statistics. Results are expressed as mean ± SE values. Statistical significance was calculated using a one-way ANOVA followed by Dunnett's test. Differences were considered significant at a level of $p < 0.05$.

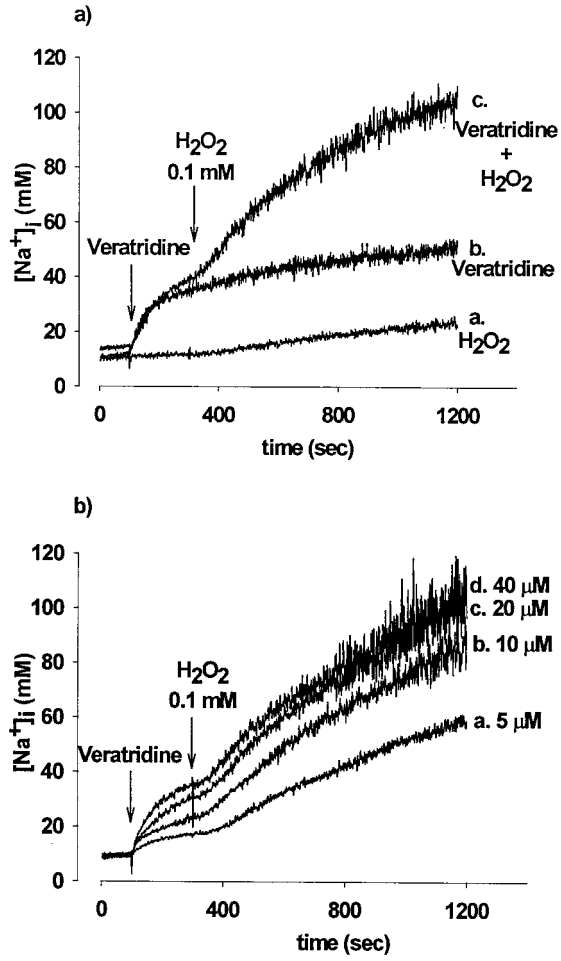

Figure 1. $[Na^+]_i$ measured in nerve terminals loaded with SBFI. *a,* H_2O_2 (0.1 mM) was added at 300 sec without previous treatment (*trace a*) or 200 sec after stimulation with 40 μM veratridine (*trace c*). *Trace b* shows the effect of veratridine applied as indicated, without subsequent addition of H_2O_2. *b,* Veratridine was added at 100 sec in 5 (*trace a*), 10 (*trace b*), 20 (*trace c*), or 40 μM concentration (*trace d*), then H_2O_2 was given in 0.1 mM concentration. Traces are representative of four independent experiments. Basal $[Na^+]_i$ was 12 ± 2.4 mM ($n = 36$). Quantitative data of these experiments are included in Figure 3.

RESULTS

The effect of oxidative stress combined with a Na^+ load on $[Na^+]_i$ homeostasis

To investigate $[Na^+]_i$ homeostasis in nerve terminals, a Na^+ load was induced by veratridine, which blocks the inactivation of voltage-dependent Na^+ channels and shifts the activation to more negative membrane potential, thereby causing persistent channel activation (Catterall, 1980). By allowing Na^+ entry via these channels, veratridine enhances $[Na^+]_i$ (Deri and Adam-Vizi, 1993) and induces depolarization and Ca^{2+} influx in nerve terminals (Adam-Vizi and Ligeti, 1986). Resting $[Na^+]_i$ in nerve terminals was 12 ± 2.4 mM ($n = 36$), and Figure 1*a* indicates that with the addition of 40 μM veratridine $[Na^+]_i$ started to increase and attained a stable elevated level within a few minutes. $[Na^+]_i$ at the end of a 20 min incubation period with 40 μM veratridine was 43 ± 3.1 mM ($n = 1$ 4). Oxidative stress induced by H_2O_2 (0.1 mM) alone caused only a slow and relatively small increase in $[Na^+]_i$ reaching 21 ± 1.3 mM ($n = 1$ 4) over an incubation period of 20 min (Fig. 1*a*, *trace a*) (see also Tretter and Adam-Vizi,

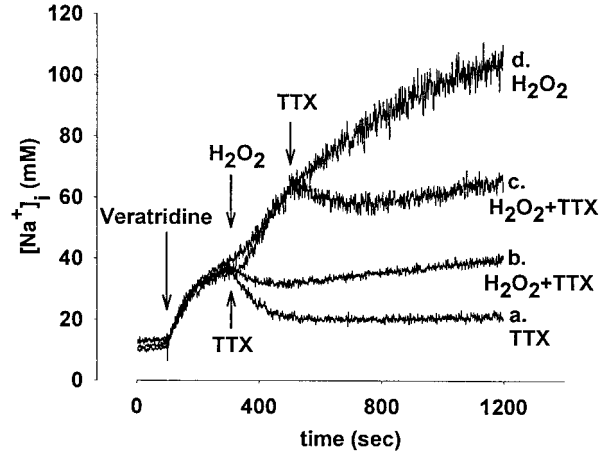

Figure 2. The effect of TTX on $[Na^+]_i$ given after veratridine in the absence or presence of H_2O_2. Veratridine (40 μM) was applied at 100 sec, then 1 μM TTX (*trace a*) or TTX + 0.5 mM H_2O_2 (*b*) was applied at 300 sec. For *trace c*, TTX was applied at 500 sec. For *trace d*, veratridine and H_2O_2 were added as indicated without TTX. Traces are representative of three independent experiments made in duplicate.

1996). When H_2O_2 was applied subsequently to veratridine, a large additional increase in $[Na^+]_i$ was induced, which was continuous, and no new $[Na^+]_i$ equilibrium was attained. It is remarkable that a modest initial $[Na^+]_i$ load was sufficient for the subsequent oxidative stress to induce a large additional increase in $[Na^+]_i$ (Fig. 1*b*). When $[Na^+]_i$ was higher by only a few millimolar concentration (18 ± 2 mM; n = 1 4) at the time of the oxidant application (in the presence of 5 μM veratridine), $[Na^+]_i$ was greatly enhanced by 0.1 mM oxidant and reached 58 ± 5 mM (n = 1 4) by the end of a 20 min recording period (Fig. 1*b*), and the higher the initial $[Na^+]_i$, the larger was the extent of the oxidant-induced $[Na^+]_i$.

It has been reported that under exposure to veratridine mitochondrial respiration is accelerated to produce sufficient amount of ATP for the Na,K–ATPase (Pastuszko et al., 1981), enabling a new $[Na^+]_i$ equilibrium at an elevated level to be sustained. The question arises whether an additional $[Na^+]_i$ rise in the presence of the oxidant could be the result of an impaired extrusion of Na^+ from the cytosol by the Na,K–ATPase. This was examined by the application of tetrodotoxin (TTX; 1 μM) at different time points to block Na^+ entry via voltage-operated Na channels (Fig. 2). After addition of TTX subsequent to stimulation with veratridine, $[Na^+]_i$ started to decrease from an elevated level (38 ± 3 mM; n = 6) and returned close to the baseline level (18 ± 2.3 mM; n = 6; measured 5 min after addition of TTX), reflecting the restoration of the normal Na^+ equilibrium caused by extrusion of Na^+ by the Na,K–ATPase (Fig. 2, *trace a*). When TTX was applied together with the oxidant (Fig. 2, *trace b*), $[Na^+]_i$ was only slightly decreased immediately after the application of TTX, but then remained at an elevated level (37 ± 3.1 mM; n = 6; measured 5 min after addition of TTX). Likewise, TTX given 200 sec after the oxidative challenge, at an even higher $[Na^+]_i$ (65 ± 4 mM; n = 6) (Fig. 2, *trace c*), prevented further increase in $[Na^+]_i$, but $[Na^+]_i$ showed no tendency of returning to the baseline level (64 ± 3.7 mM; n = 6; measured at the end of the 20 min incubation). These results indicate the inability of the Na,K–ATPase to reestablish normal $[Na^+]_i$ from an elevated level during exposure to an oxidative insult.

Table 1. ATP and [ATP]/[ADP] ratio in the presence of H_2O_2 and veratridine

	ATP nmol/mg	[ATP]/[ADP]
Control	3.76 ± 0.003	7.3 ± 0.23
Veratridine	2.95 ± 0.13*	6.7 ± 0.26
H_2O_2	2.4 ± 0.09*	3.77 ± 0.09*
Veratridine + H_2O_2	0.52 ± 0.03*[a]	0.87 ± 0.06*[a]
Veratridine + H_2O_2 (no Ca^{2+})	0.48 ± 0.04*	0.86 ± 0.04*
Ouabain	3.70 ± 0.05	7.24 ± 0.17
Ouabain + H_2O_2	1.77 ± 0.09*[a]	3.09 ± 0.20*[a]
Veratridine + ouabain	3.7 ± 0.03	8.19 ± 0.15*
Veratridine + H_2O_2 + ouabain	1.24 ± 0.04*[b]	2.72 ± 0.18*[b]

Synaptosomes were incubated for 12 min in standard medium, and ATP level and [ATP]/[ADP] ratio were determined at the end of the incubation. Additions were as described for Figure 1. H_2O_2 (0.5 mM) was applied at 300 sec after addition of veratridine (40 μM) at 100 sec, where indicated. Ouabain (500 μM) was given at 50 sec. "No Ca^{2+}" indicates that the experiment was performed in a medium containing no added Ca^{2+} and 100 μM EGTA. The control ATP level (without any addition) measured in a Ca^{2+}-free medium was not significantly different (3.85 ± 0.04 nmol/mg; n = 4) from the control shown in the Table. Data are the average of four experiments ± SE (n = 4).

*Significantly different compared with the respective control values.

[a]Significantly different compared with values obtained with veratridine or H_2O_2 alone.

[b]Significantly different compared with the value obtained with veratridine + H_2O_2 ($p < 0.001$).

ATP depletion caused by a combined action of H_2O_2-induced oxidative stress and $[Na^+]_i$ rise: correlation between ATP depletion and deregulation of $[Na^+]_i$

Next we wanted to examine whether an insufficient ATP supply could be responsible for the failure of the Na,K–ATPase in the combined presence of oxidative stress and a $[Na^+]_i$ load.

It has been reported that incubation of synaptosomes with veratridine leads to a decrease in the ATP content attributable to stimulation of the Na,K–ATPase caused by an increase in $[Na^+]_i$ (Erecinska and Dagani, 1990; Erecinska et al., 1996). We have shown recently that H_2O_2 decreases NADH production in the citric acid cycle, thus limiting the respiratory capacity in nerve terminals (Chinopoulos et al., 1999), and, consistent with this, decreasing the ATP content (Tretter et al., 1997). The possibility emerges from these observations that mitochondria with an impaired respiratory capacity during oxidative stress may not be able to generate a sufficient amount of ATP to fuel the Na,K–ATPase under an increased demand created by a small rise in $[Na^+]_i$. Table 1 indicates that indeed there was a drastic fall in the ATP content and [ATP]/[ADP] ratio when nerve terminals were challenged with H_2O_2 during stimulation with veratridine. The control ATP content corresponds to 1.56 mM ATP concentration in the synaptoplasm (calculated with a cytosolic volume of 2.4 μl/mg protein; Adam-Vizi and Ligeti, 1984) being in good agreement with data previously reported for this preparation (Kauppinen and Nicholls, 1986) (see also Erecinska et al., 1996). It should be mentioned that this ATP level and [ATP]/[ADP] ratio is somewhat smaller than those measured in cultured cells (Silver et al., 1997) or different tissues (Erecinska and Wilson, 1982), although great variations can occur in the [ATP]/[ADP] ratio depending on the activity of the tissues (Erecinska and Wilson, 1982). The low ATP level (0.52 ± 0.03 nmol/mg; corresponding to 216 μM in the synaptoplasm), reached 7 min after application of the oxidative insult, was stable, and no further decrease was seen over an incubation for 20 min (data not shown). The observation that veratridine itself induces a ~25% decrease in ATP

level, which could be prevented by preincubation with ouabain, agrees with the findings of Erecinska and Dagani (1990). It is important to note that the ATP depletion induced by H_2O_2 and veratridine was independent of extracellular [Ca^{2+}] as in the absence of Ca^{2+} a similar, very low level of ATP (0.48 ± 0.04 nmol/mg; $n = 4$) was measured. It is also demonstrated in Table 1 that ouabain, which could prevent the excessive utilization of ATP by the Na,K–ATPase, significantly attenuated both the ATP loss (1.24 ± 0.04 vs 0.52 ± 0.03 nmol/mg protein) and the decrease in the [ATP]/[ADP] ratio (2.72 ± 0.18 vs 0.87 ± 0.06 nmol/mg) induced by veratridine plus H_2O_2. The restoration of ATP under this condition was significant, but ouabain failed to fully protect ATP to the level seen with H_2O_2 alone. This may be related to the effect of ouabain on the ATP level in the presence of H_2O_2 (1.77 ± 0.09 nmol/mg). The mechanism for this is unclear but may be the result of an altered [Na^+]$_i$ and [K^+]$_i$, which, together with an inhibition of the TCA cycle by H_2O_2 (Chinopoulos et al., 1999), could result in a larger decrease in the ATP level.

These results strongly suggest that compromised mitochondria under oxidative stress are unable to balance an increased ATP demand created by the stimulation of the Na^+ pump caused by an increase in [Na^+]$_i$. Therefore, the sustained Na^+ load, which itself results in a stable [Na^+]$_i$ rise, when it has an oxidative insult superimposed on it, could produce a vicious cycle in which the initial [Na^+]$_i$ load, by stimulating the Na,K–ATPase, leads to an ATP depletion which, in turn, restricts extrusion of Na^+, leading to an additional increase in [Na^+]$_i$. Data shown in Figure 3 appear to reinforce this interpretation. The rise in [Na^+]$_i$ induced by H_2O_2 in veratridine-treated nerve terminals was remarkably parallel with a decrease in the ATP level (Fig. 3). In agreement with a previous report (Erecinska and Dagani, 1990), veratridine itself (5–40 μM) caused only a small change in the ATP level; in fact, significant decrease was only observed in the presence of 40 μM veratridine (Fig. 3*b*). However, the addition of H_2O_2 (0.1 or 0.5 mM) induced a large decrease in the ATP level and, parallel with this, higher increases in [Na^+]$_i$ (Fig. 3).

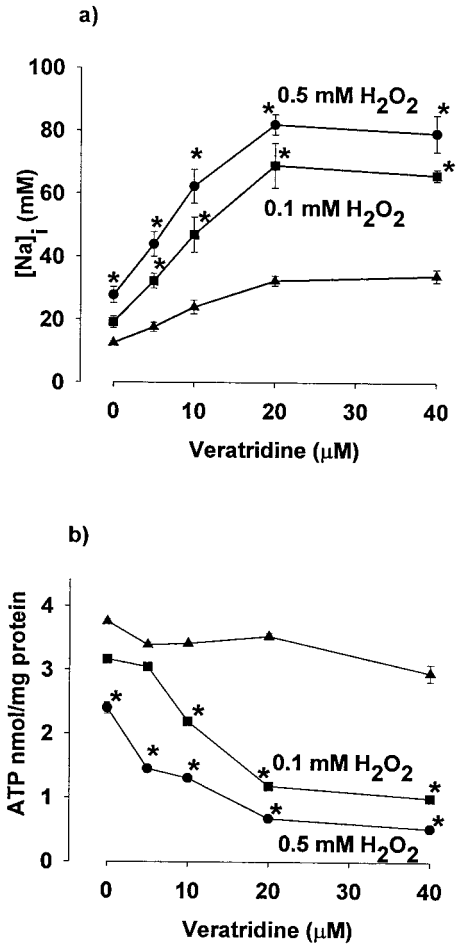

Figure 3. H_2O_2 induced increase in [Na^+]$_i$ and decrease in [ATP]. Additions were as described for Figure 1. Veratridine was given in different concentrations without further addition (▲) or followed by treatment with H_2O_2 at 300 sec in 0.1 mM (■) or 0.5 mM (●) concentrations. [Na^+]$_i$ (*a*) and ATP level (*b*) measured at 720 sec in parallel samples are shown as a function of veratridine concentrations. Data are the average of four determinations ± SE. SE is not shown where it is smaller than the symbol. *Significantly different from data obtained with veratridine alone.

Collapse of $\Delta\psi m$ in the presence of veratridine and H_2O_2

Given the ATP depletion caused by the combined presence of oxidative stress and a [Na^+]$_i$ load, we wanted to investigate the state of mitochondria under this condition. For this, $\Delta\psi m$ was measured *in situ* by monitoring the fluorescence of JC-1 at 535 nm. Figure 4, trace b, shows that JC-1 monomer fluorescence was only marginally and transiently increased in the presence of 40 μM veratridine, indicating that plasma membrane depolarization and an increase in [Na^+]$_i$ have no significant influence on $\Delta\psi m$. Application of veratridine in higher concentrations (up to 80 μM) gave essentially the same result (data not shown). We have reported recently that H_2O_2 itself has no effect on $\Delta\psi m$ (Chinopoulos et al., 1999), which is also demonstrated in Figure 4, trace a. However, when H_2O_2 (0.1–0.5 mM) was applied to nerve terminals depolarized previously by veratridine (40 μM), an increase in the monomer fluorescence was observed that was proportional to the concentrations of H_2O_2 (Fig. 4, *traces c–e*). A concentration of 0.5 mM H_2O_2 applied after veratridine nearly completely collapsed $\Delta\psi m$ over an incubation period of 20 min. The effect of H_2O_2 on $\Delta\psi m$ was dependent on Ca^{2+}; in the absence of extracellular Ca^{2+} (no added Ca^{2+} + 100 μM EGTA present in the medium), addition of H_2O_2 subsequent to veratridine produced a significantly attenuated change in $\Delta\psi m$ (Fig. 5), suggesting that the effect of H_2O_2 on $\Delta\psi m$ was associated with a rise in [Ca^{2+}]$_i$. Pretreatment of synaptosomes with 10 μM cyclosporin A had no influence on the collapse of $\Delta\psi m$ induced by veratridine and H_2O_2 (data not shown).

We addressed the question whether depolarization of the plasma membrane, or alternatively an increase in [Na^+]$_i$ induced by veratridine, plays a role in the loss of $\Delta\psi m$ by H_2O_2 when added after veratridine. To resolve this, we applied an alternative means to depolarize nerve terminals, using high [K^+], which activates voltage-operated calcium channels (VOCCs), giving rise to a [Ca^{2+}]$_i$ signal (Ashley et al., 1984) without inducing any change in [Na^+]$_i$ (Deri and Adam-Vizi, 1993). Figure 5 shows that 40 mM [K^+] itself did not influence $\Delta\psi m$, and H_2O_2 (0.5 mM) added 200 sec after K^+ had only a marginal effect. This is in marked contrast to what was observed when H_2O_2 was added after veratridine, in spite of a larger depolarization induced by 40 mM K^+ (~43 mV) than that caused by 40 μM veratridine (~28 mV; Adam-Vizi and Ligeti, 1984). These results suggest that plasma membrane depolarization, even when sustained for 20 min, has no influence on $\Delta\psi m$; in contrast, an increase in [Na^+]$_i$

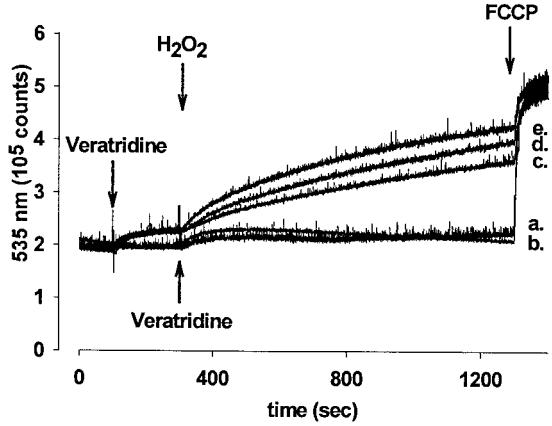

Figure 4. Fluorescence of JC-1 at 535 nm in the presence of veratridine and H_2O_2. Synaptosomes loaded with JC-1 were incubated in a standard medium (0.2 mg/ml). Veratridine (40 μM) was added at 100 sec followed by addition of H_2O_2 at 300 sec in 0.1 mM (*trace c*), 0.2 mM (*trace d*), or 0.5 mM concentrations (*trace e*). *Traces a* and *b* show the effects of 0.5 mM H_2O_2 (*a*) and 40 μM veratridine (*b*), respectively, given at 300 sec. Traces are representative of four independent experiments. A 1 μM concentration of FCCP was added at the end of each experiment to generate a signal representing the total collapse of $\Delta\psi m$. Quantitative data of these experiments are included in Table 2.

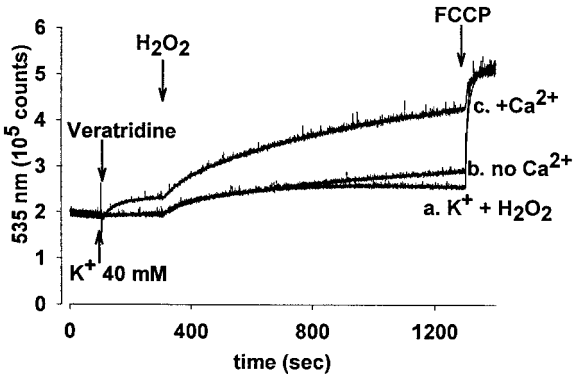

Figure 5. The combined effect of veratridine and H_2O_2 on $\Delta\psi m$ is dependent on the presence of Ca^{2+} in the medium. JC-1 fluorescence at 535 nm in response to veratridine (40 μM) and H_2O_2 (0.5 mM) was measured in synaptosomes in the presence of 2 mM Ca^{2+} (*trace c*) or when Ca^{2+} was lacking in the medium (no Ca^{2+} was added, and 100 μM EGTA was present) (*trace b*). *Trace a* shows the effect of 0.5 mM H_2O_2 (300 sec) added subsequent to 40 mM K^+ (100 sec). Traces are representative of three experiments. Quantitative data of these experiments are included in Table 2.

appears to have a great impact on the state of mitochondria subsequently exposed to an oxidative insult. It is important to note that increase in $[Na^+]_i$ itself, in the absence of oxidative stress, has no effect on $\Delta\psi m$; even very high $[Na^+]_i$ alone, in the presence of 500 μM ouabain and 40 μM veratridine (~70–80 mM), was without significant effect on $\Delta\psi m$ (data not shown).

A statistical summary of the results shown in Figures 4 and 5 is given in Table 2, indicating that H_2O_2 in combination with veratridine significantly increased the fluorescence of JC-1 at 535 nm. A large part of this required the presence of Ca^{2+} in the medium; in the absence of Ca^{2+} the increase in the fluorescence, although statistically significant, was marginal.

Table 2. Statistical analysis of data on fluorescence of JC-1 at 535 nm in the presence of veratridine and H_2O_2

	No H_2O_2	H_2O_2
Control	2.07 ± 0.04	2.11 ± 0.01 (0.5 mM)
Veratridine	2.20 ± 0.03	3.57 ± 0.02 (0.1 mM)*
Veratridine	2.20 ± 0.03	3.95 ± 0.02 (0.2 mM)*
Veratridine	2.20 ± 0.03	4.30 ± 0.01 (0.5 mM)*[a]
Veratridine (no Ca^{2+})	2.30 ± 0.05	2.75 ± 0.02 (0.5 mM)*[a]
K^+	2.10 ± 0.02	2.35 ± 0.05 (0.5 mM)*

Values of relative fluorescence (10^5 counts) monitored in the experiments presented in Figures 4 and 5 at 1000 sec are shown. Veratridine (40 μM) or KCl (40 mM) was added at 100 sec without further addition, or H_2O_2 was given at 300 sec at concentrations indicated in parentheses. Data are average ± SE values of three (for K^+ and "no Ca^{2+}") or four experiments.

*Significantly different compared with the control value ($p < 0.001$).

[a]Significantly different compared with one another ($p < 0.001$).

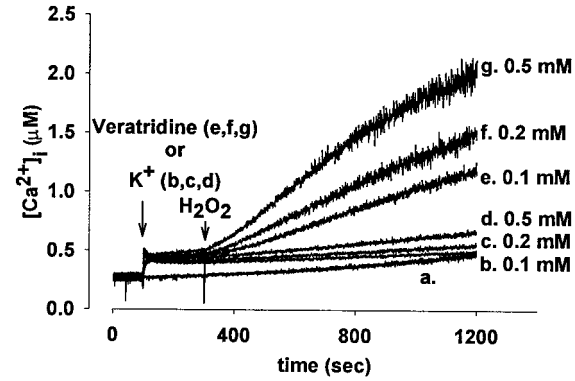

Figure 6. $[Ca^{2+}]_i$ measured in synaptosomes loaded with fura-2. Nerve terminals were depolarized by 40 mM K^+ (*traces b–d*) or 40 μM veratridine (*traces e–g*) applied at 100 sec, then H_2O_2 was added in 0.1, 0.2, or 0.5 mM concentrations as indicated. *Trace a* shows the effect of 0.5 mM H_2O_2 given at 100 sec. Traces are representative of four determinations.

Table 3. $[Ca^{2+}]_i$ 5 or 15 min after addition of H_2O_2 in the presence of veratridine

	$[Ca^{2+}]_i$ nM	
	5 min	15 min
Control	280 ± 41	307 ± 36
Veratridine 40 μM	450 ± 45	475 ± 30
+H_2O_2 0.1 mM	770 ± 53*	1353 ± 156*[a]
+H_2O_2 0.2 mM	911 ± 85*[a]	1549 ± 130*[a]
+H_2O_2 0.5 mM	1118 ± 44*[a]	2100 ± 61*[a]

Experimental conditions were as described for Figure 6. Veratridine was added at 100 sec, followed by addition of H_2O_2 at 300 sec. $[Ca^{2+}]_i$ values obtained at 600 or 1200 sec (5 or 15 min after application of H_2O_2) are shown. Values (±SE) are average of four independent determinations ($n = 4$).

*Significantly different from the respective control values.

[a]Significantly different from the value obtained with veratridine alone ($p < 0.001$).

$[Ca^{2+}]_i$ rise in the presence of veratridine and H_2O_2 is parallel with a decrease in $\Delta\psi m$

The question arises why $\Delta\psi m$ collapses under oxidative stress when combined with a $[Na^+]_i$ load and what is reflected in the Ca^{2+}-dependent character of the loss of $\Delta\psi m$.

To determine whether oxidative stress could enhance the veratridine-evoked $[Ca^{2+}]_i$ increase accounting for the collapse of $\Delta\psi m$, $[Ca^{2+}]_i$ was measured under identical conditions to those

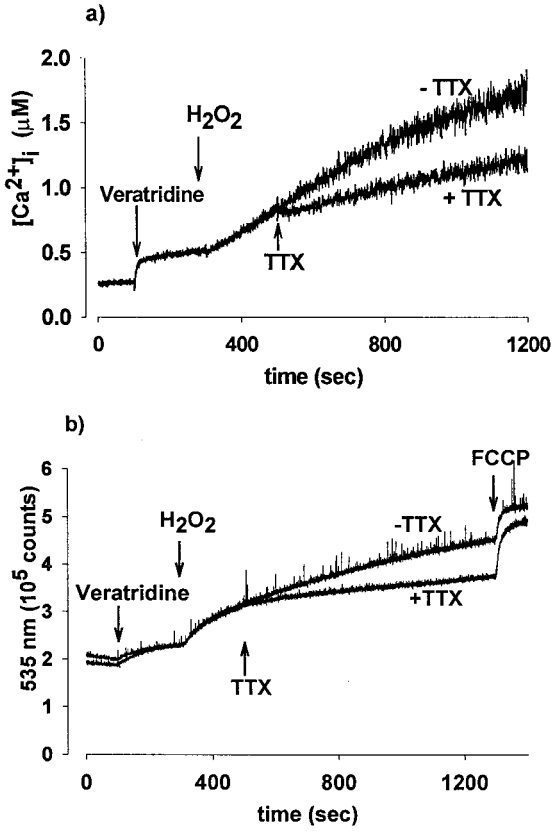

Figure 7. $[Ca^{2+}]_i$ rise and depolarization of $\Delta\psi m$ induced by H_2O_2 (0.5 mM) and veratridine (40 μM) is diminished after addition of TTX. Veratridine and H_2O_2 were added as indicated, then 200 sec after H_2O_2, TTX (1 μM) was applied, and $[Ca^{2+}]_i$ (*a*) and JC-1 fluorescence (*b*) were measured in parallel samples loaded with fura-2 or JC-1. Traces are representative of three independent experiments.

used in the experiments for monitoring $\Delta\psi m$. We found (Fig. 6, Table 3) that veratridine (40 μM) induced a moderate rise in $[Ca^{2+}]_i$ and, similarly, H_2O_2 itself caused only a slow and small increase in $[Ca^{2+}]_i$ (White and Clarke, 1988) (see also Tretter and Adam-Vizi 1996). However, after addition of H_2O_2 (0.1–0.5 mM) 200 sec after stimulation with veratridine, $[Ca^{2+}]_i$ started to increase further, and by the end of an incubation for 20 min $[Ca^{2+}]_i$ reached 2100 ± 61 nM in the presence of 0.5 mM H_2O_2 (Fig. 6, *traces e–g*, Table 3). This is likely to be an underestimated value, given the low K_m of fura-2 for Ca^{2+} (Hyrc et al., 1997). The rise in $[Ca^{2+}]_i$ induced by H_2O_2 and $[Na^+]_i$ load was unaltered by pretreatment with 10 μM cyclosporin A (data not shown). The rate of change of $[Ca^{2+}]_i$ in the presence of veratridine and H_2O_2 is very similar to that of $\Delta\psi m$ shown in Figure 4. This, and the Ca^{2+} dependency of the decrease in $\Delta\psi m$ by H_2O_2 shown in Figure 5, suggest that depolarization of mitochondria is related to an enhanced $[Ca^{2+}]_i$ rise induced by the oxidant in Na^+-loaded nerve terminals. Consistent with this, H_2O_2 applied 200 sec after plasma membrane depolarization by 40 mM K^+, a condition resulting in no change of $\Delta\psi m$ (Fig. 5, *trace a*), failed to induce a significant increase in $[Ca^{2+}]_i$ (Fig. 6).

The correlation between an enhanced $[Ca^{2+}]_i$ rise and a fall in $\Delta\psi m$ was reinforced by the effect of TTX. Addition of TTX (1 μM) to inhibit voltage-dependent Na^+ channels 200 sec after imposition of the oxidative stress (Fig. 7) significantly attenuated the H_2O_2-induced $[Ca^{2+}]_i$ rise; $[Ca^{2+}]_i$ increased from 820 ± 30 to 1110 ± 50 nM ($n = 3$) during the incubation period with TTX, whereas over a same period of incubation without TTX $[Ca^{2+}]_i$ reached 1910 ± 70 nM ($n = 3$). Parallel with changes in $[Ca^{2+}]_i$, the decrease in $\Delta\psi m$ induced by H_2O_2 was also attenuated by TTX (Fig. 7*b*). This also indicates that Na^+ entry is a critical factor both in the large increase of $[Ca^{2+}]_i$ and in the mitochondrial depolarization occurring in the combined presence of veratridine and H_2O_2. These results suggest that the collapse of $\Delta\psi m$ is very likely to result from a large increase in $[Ca^{2+}]_i$ occurring when oxidative stress is superimposed on a $[Na^+]_i$ load.

Basis for increase in $[Ca^{2+}]_i$

Figure 6 and Table 3 indicate that oxidative stress and $[Na^+]_i$ load initiate a large increase in Ca^{2+}, which does not attain a new equilibrium, but rather exhibits the tendency of a continuous, uncontrolled $[Ca^{2+}]_i$ rise. The question arises as to what the underlying mechanism for this apparent Ca^{2+} deregulation could be.

Entry via VOCCs

The $[Ca^{2+}]_i$ signal after depolarization by high $[K^+]$ is the result of activation of VOCCs followed by a rapid inactivation (Ashley et al., 1984; Alvarez Maubecin et al., 1995), thus the lack of effect of H_2O_2 on $[Ca^{2+}]_i$ applied after K depolarization shows that H_2O_2 has no effect on VOCCs under these conditions. This was also indicated by the results that both $[Ca^{2+}]_i$ rise and mitochondrial depolarization elicited by H_2O_2 in Na^+-loaded synaptosomes were unaltered by pretreatment with inhibitors of N-, P-, Q- or L-type Ca^{2+} channels (ω-conotoxin, 1 μM; ω-agatoxin IVA, 50 nM; ω-conotoxin MVIIC, 1 μM; and tai-conotoxin, 160 nM, respectively; $n = 3$; data not shown). When nerve terminals incubated in Ca^{2+}-free medium were challenged with veratridine plus the oxidant, no change in $[Ca^{2+}]_i$ was produced (data not shown). These results indicate that extracellular Ca^{2+} is involved in the oxidant-induced $[Ca^{2+}]_i$ rise, but no Ca^{2+} entry is likely to be mediated by VOCCs.

This latter finding requires a comment, because it has been shown recently that $[Ca^{2+}]_i$ signal is enhanced when high $[K^+]$ is applied in the presence of the oxidant (Tretter et al., 1997), and consistent with this, H_2O_2 has been reported to enhance Ca^{2+} influx via VOCCs (Li et al., 1998). The lack of effect of the oxidant on $[Ca^{2+}]_i$ applied after the depolarizing stimulus in this study might indicate that oxidative conditions should be present at the onset of the activation of VOCCs for the Ca^{2+} influx to be enhanced, and addition of H_2O_2 after the stimulus, even during a sustained depolarization, is no longer able to influence Ca^{2+} influx.

Activation of glutamate receptors

We also considered whether glutamate, which is assumed to be released from nerve terminals when stimulated with veratridine and H_2O_2, could contribute to the increase in $[Ca^{2+}]_i$, but neither the NMDA receptor antagonist MK 801 (10 μM) nor GYKI 52466 (50 μM), blocker of the AMPA receptors had any influence on the $[Ca^{2+}]_i$ rise induced by 40 μM veratridine and 0.5 mM H_2O_2 (data not shown).

Effect of mitochondrial depolarization

Collapse of $\Delta\psi m$ in many cells gives rise to an elevated $[Ca^{2+}]_i$ owing to an impaired buffering of Ca^{2+} by mitochondria (Budd and Nicholls, 1996; Wang and Thayer, 1996; White and Reynolds, 1996). In nerve terminals no change in $[Ca^{2+}]_i$ could be observed

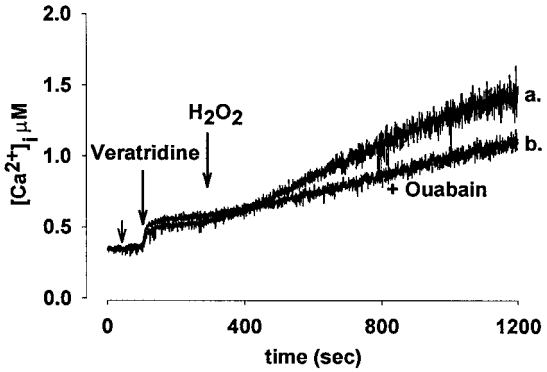

Figure 8. $[Ca^{2+}]_i$ rise induced by veratridine and H_2O_2 in the presence or absence of ouabain. Veratridine (40 μM) and H_2O_2 (0.2 mM) were added as indicated (*a*), and $[Ca^{2+}]_i$ was measured in fura-2-loaded nerve terminals. Ouabain (500 μM) was given 50 sec before veratridine (*b*), as indicated by the *arrow*. Traces are representative of three experiments. Quantitative data are given in Table 4.

after dissipation of $\Delta\psi m$ by rotenone (2 μM)/oligomycin (10 μM) either in the presence or in the absence of extracellular Ca^{2+} (data not shown), thus it is unlikely that $[Ca^{2+}]_i$ rise induced by veratridine and H_2O_2 could be secondary to a loss of $\Delta\psi m$.

Inhibition of plasmalemmal Ca^{2+}–ATPase

Given the severe ATP depletion induced by H_2O_2 in the presence of veratridine, it is possible to propose that the large increase in $[Ca^{2+}]_i$ under this condition results from an impaired ATP-dependent Ca^{2+} removal, primarily by the plasmalemmal Ca^{2+}–ATPase. This prediction was supported by experiments with ouabain, which is able to preserve a significant part of ATP during stimulation with veratridine and H_2O_2 (Table 1). Figure 8 shows that in the simultaneous presence of ouabain (500 μM) and veratridine (40 μM), $[Ca^{2+}]_i$ increased to a slightly higher level compared to that observed with veratridine alone. This is in agreement with a higher $[Na^+]_i$ possibly driving more Ca^{2+} into the terminals via the Na^+–Ca^{2+} exchanger known to be present in the plasma membrane of nerve terminals (Gill, 1982; Sanchez-Armass and Blaustein, 1987). The presence of ouabain along with veratridine attenuated the H_2O_2-induced increase in $[Ca^{2+}]_i$ (Fig. 8). The data in Table 4 show that ouabain attenuated $[Ca^{2+}]_i$ rise induced by H_2O_2 in Na^+-loaded synaptosomes by ~50%. The remarkable correlation between the fall in [ATP] and the rise in $[Ca^{2+}]_i$ suggests that inhibition of the ATP-dependent removal of $[Ca^{2+}]_i$ by the Ca^{2+}–ATPase in the plasmalemma is the cause of the increased $[Ca^{2+}]_i$. The evidence argues against the involvement of Ca^{2+} entry via reversal of the plasmalemma Na^+–Ca^{2+} exchanger. Although 40 μM veratridine + 500 μM ouabain increased $[Na^+]_i$ to 70–80 mM within a few minutes (data not shown), the Ca^{2+} rise was only slightly higher than that caused by veratridine alone, where $[Na^+]_i$ rose only to 40 mM (Fig. 8). However, even this result does not entirely rule out the possibility that when the ATP level is also reduced simultaneously, as observed with veratridine plus H_2O_2 (but not with veratridine plus ouabain), the reverse function of the Na^+–Ca^{2+} exchanger could become significant. Unfortunately experiments with inhibitors of the Na^+–Ca^{2+} exchanger (Bepridil, 10 μM; 3′,4′-dichlorobenzamil; 10 μM) gave ambiguous results as Bepridil appears to interfere with Na^+ channels and prevent the effect of veratridine on $[Na^+]_i$, and 3′,4′-dichlorobenzamil gives fluorescent signals at the wavelengths used for measuring $[Ca^{2+}]_i$ and $[Na^+]_i$ (data not shown).

DISCUSSION

The major observation in the present study is that when oxidative stress occurs together with a Na^+ load, the damaging effect of oxidative stress is greatly exacerbated. A key element in the dysfunction is a large fall in [ATP] in the combined presence of H_2O_2 and Na^+ load. The basis for this is an increased utilization of ATP by the Na^+ pump activated by a Na^+ entry coupled with the inability of mitochondria to respond adequately with increasing ATP production because of limitation of the respiratory capacity by H_2O_2. This leads to a vicious cycle in which the $[Na^+]_i$ increase augments the decrease in [ATP], which, in turn, further inhibits the Na^+ pump, enhancing the $[Na^+]_i$ increase. The large fall in ATP gives rise to the inability to remove cytosolic Ca^{2+} via the plasmalemmal ATPase. The resulting Ca^{2+} accumulation then leads to a collapse of $\Delta\psi m$.

A crucial effect of H_2O_2 in the early stage of the oxidative insult appears to be the inhibition of α-ketoglutarate dehydrogenase and, as a consequence, a decrease in the mitochondrial NADH production (Chinopoulos et al., 1999). The ATP level under this condition, although decreased (Tretter et al., 1997), is still adequate to secure a resting function of the ATP-driven ion pumps in the plasma membrane, thus, the Na^+ and Ca^{2+} electrochemical gradients are only slightly decreased (Tretter and Adam-Vizi, 1996), and $\Delta\psi m$ is maintained (Chinopoulos et al., 1999).

When oxidative stress is imposed on nerve terminals in which $[Na^+]_i$ is increased, a complex dysfunction develops with (1) a drastic fall in the ATP level, (2) a deregulation of $[Na^+]_i$ and $[Ca^{2+}]_i$, and (3) the loss of $\Delta\psi m$. In the interpretation of these observations, the following questions have to be addressed: (1) what are the underlying mechanisms and the sequence of these changes?, and (2) what is their relevance to pathological conditions in which oxidative stress is assumed to have a pivotal role?

Fall in the ATP level

H_2O_2 when given alone, produces not more than ~30% decrease in the ATP level after incubation for 7 min (Table 1; Tretter et al., 1997). However, owing to a combined effect of oxidative stress and Na^+ load, the energy resources of nerve terminals are almost completely drained (Table 1, Fig. 3). The most obvious explanation for the development of the severe energy deficit is that mitochondria working with a limited respiratory capacity under oxidative stress (Chinopoulos et al., 1999) are unable to produce sufficient amount of ATP when an additional demand presents itself because of stimulation of the Na,K–ATPase by an increased $[Na^+]_i$. Preliminary experiments using different mitochondrial inhibitors together with a Na^+ load appear to be consistent with this interpretation (data not shown).

It is important to note that the energy deficit brought about by H_2O_2 when applied at an elevated $[Na^+]_i$ is unrelated to an increase in $[Ca^{2+}]_i$ (Fig. 6, Table 1). This strongly suggests that the decrease in ATP level is upstream from the large increase in $[Ca^{2+}]_i$. Since, in the absence of Ca^{2+}, oxidative stress together with a Na^+ load deplete ATP at a sustained $\Delta\psi m$, the loss of ATP should be also upstream from the collapse of $\Delta\psi m$.

Dissipation of $[Na^+]$ and $[Ca^{2+}]$ gradients

H_2O_2, when applied after veratridine, at an elevated $[Na^+]_i$, induced a large additional increase in $[Na^+]_i$ (Fig. 1*a,b*). It appears most likely that, owing to the initial rise in $[Na^+]_i$

Table 4. Comparison of the effect of H_2O_2 on $[Ca^{2+}]_i$ in the presence of veratridine with or without ouabain

	5 min		15 min	
	$[Ca^{2+}]_i$ nM	$\Delta[Ca^{2+}]_i$ nM	$[Ca^{2+}]_i$ nM	$\Delta[Ca^{2+}]_i$ nM
Veratridine	418 ± 39		430 ± 41	
$+H_2O_2$	866 ± 51	448 ± 31*	1466 ± 102	1036 ± 62[a]
Veratridine + Ouabain	530 ± 10		550 ± 25	
$+H_2O_2$	733 ± 11	203 ± 20*	1000 ± 70	450 ± 60[a]

Experimental conditions were as described for Figure 8. Veratridine (40 μM) was added at 100 sec, without further treatment (not shown in Fig. 5), or followed by addition of H_2O_2 (0.2 mM) at 300 sec. Ouabain (500 μM) was given 50 sec before veratridine where indicated. $[Ca^{2+}]_i$ values (±SE) obtained at 600 or 1200 sec (5 or 15 min after application of H_2O_2) in three independent experiment are shown ($n = 3$). Basal $[Ca^{2+}]_i$ was 260 ± 25 nM.

*[a]Significantly different as compared with one another ($p < 0.001$).

produced by veratridine, the Na,K–ATPase is already stimulated at the onset of the application of oxidative stress, but as a result of the effect of the oxidant on the mitochondria, as discussed above, ATP production becomes insufficient. This, in turn, would limit the function of the Na,K–ATPase, resulting in an additional gradual rise in $[Na^+]_i$. At an ATP level of 0.52 ± 0.03 nmol/mg (216 μM) and with an [ATP]/[ADP] ratio reduced to 10% of the control (Table 1), Na^+ extrusion by the Na,K–ATPase should be severely impaired (K_m for ATP is 200–400 μM; Erecinska and Dagani, 1990), accounting for the gradual collapse of the $[Na^+]$ gradient. Therefore the picture of a vicious cycle emerges, in which a relatively small Na^+ load aggravates the effect of oxidative stress creating gradually an energy deficit, which in turn leads to $[Na^+]_i$ deregulation.

To this picture, another element, a large increase in $[Ca^{2+}]_i$ induced by the combination of oxidative stress and $[Na^+]_i$ load should also be added (Fig. 6, Table 3). The increase is not caused by activation of VOCCs or glutamate receptors because antagonists to these pathways did not affect $[Ca^{2+}]$ rise (data not shown).

It has been suggested that Ca^{2+} entry into rat optic nerves during anoxia is mediated by a reverse Na^+–Ca^{2+} exchange (Stys et al., 1992). This seems unlikely to be the mechanism for the $[Ca^{2+}]_i$ increase in this study because when $[Na^+]_i$ was increased to 70–80 mM by the combination of ouabain and veratridine there was only a very small rise in $[Ca^{2+}]_i$. A caveat here is that the large fall in [ATP] when H_2O_2 and veratridine are used may in some way activate Ca^{2+} entry via this pathway. Such an effect of ATP has been described for the Na^+–H^+ exchanger, which has a decreased affinity to H^+ when the level of ATP is decreased (Orlowski and Grinstein, 1997).

A possible interpretation consistent with our observations is that $[Ca^{2+}]_i$ deregulation is related to the ATP depletion evolving from the combined effects of oxidative stress and Na^+ load. It is expected that, similarly to that of the Na,K–ATPase, the function of the Ca^{2+}–ATPase in the plasma membrane becomes also severely limited because of an insufficient ATP supply. The slow pattern of the $[Ca^{2+}]_i$ rise (Fig. 6) is consistent with a $[Ca^{2+}]_i$ deregulation caused by an impaired Ca^{2+} extrusion by the Ca^{2+}–ATPase. This interpretation is reinforced by the result that ouabain, which partly prevents the loss of ATP (Table 1), significantly attenuates the $[Ca^{2+}]_i$ rise under this condition (Fig. 8).

Loss of $\Delta\psi m$

The gradual depolarization of mitochondria in response to H_2O_2 in Na^+-loaded nerve terminals is clearly a Ca^{2+}-dependent process (Fig. 5) and occurs parallel with increases in $[Ca^{2+}]_i$ (Fig. 7). It could be expected that when $[Ca^{2+}]_i$ is in the micromolar range, the mitochondrial permeability transition would be induced (Duchen et al., 1993), but we obtained no evidence for the involvement of a cyclosporin A-sensitive permeability transition in the collapse of $\Delta\psi m$. This is in contrast with the glutamate-induced mitochondrial depolarization, which is sensitive to cyclosporin A (Schinder et al., 1996; White and Reynolds, 1996). Because $\Delta\psi m$ is the driving force for Ca^{2+} uptake by mitochondria, Ca^{2+} uptake itself could discharge $\Delta\psi m$ if not balanced by H^+ extrusion (Nicholls, 1985). This is the most likely mechanism for the Ca^{2+}-dependent loss of $\Delta\psi m$ observed in the present study, given the limited capacity of the respiratory chain to maintain $\Delta\psi m$ in the presence of the oxidant (Chinopoulos et al., 1999).

The increased $[Na^+]_i$ might also potentiate the effect of Ca^{2+} on mitochondria by accelerating Ca^{2+} efflux via the Na^+–Ca^{2+} exchanger present in the mitochondria of excitable cells (Crompton et al., 1978), contributing to a futile Ca^{2+} cycling. In addition, reestablishing the Na^+ gradient across the mitochondrial inner membrane by the mitochondrial Na^+–H^+ exchange against a large $[Na^+]_i$ could also be a contributing factor in the collapse of $\Delta\psi m$.

Relevance to pathological conditions

A small Na^+ load appears to be sufficient to exacerbate the condition created by oxidative stress, which could be an important contributing factor in the dysfunction developing during excessive stimulation of NMDA receptors or during reperfusion after an anoxic period, when the oxidative insult is superimposed on a disturbed $[Na^+]$ homeostasis.

It has been demonstrated that in NMDA-stimulated cells (1) $[Na^+]_i$ is increased (Kiedrowski et al., 1994a, b), (2) $\Delta\psi m$ is lost (Budd and Nicholls, 1996; Isaev et al., 1996; Schinder et al., 1996; White and Reynolds, 1996), and (3) reactive oxygen species are produced (Lafon-Cazal et al., 1993; Dugan et al., 1995; Reynolds and Hastings, 1995; Patel et al., 1996). In the light of our observations presented here, it could be assumed that the cytoplasmic Na^+ elevation, in addition to hampering Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger (Kiedrowski et al., 1994a), could contribute to cell death by aggravating the damage caused by the oxidative component of the excitotoxic stimulus. Consistent with this could be a recent report by Scanlon and Reynolds (1998) that exposure of forebrain neurons to hydrogen peroxide potentiated the mitochondrial depolarization caused by glutamate and that by Strijbos et al. (1996) suggesting that a TTX-sensitive Na^+ entry is

part of a vicious cycle that leads to neurodegeneration after stimulation of NMDA receptors.

It is well documented that oxygen–glucose deprivation induces $[Na^+]_i$ deregulation (Hansen, 1985; Stys et al., 1992; Waxman et al., 1994; for review, see Urenjak and Obrenovitch, 1996), which further worsens during reperfusion (Rose et al., 1998; Taylor et al., 1999). Reperfusion injury is generally thought to be associated with an increased production of reactive oxygen species (Cao et al., 1988; Halliwell, 1992), and consistent with this, presence of H_2O_2 at a concentration of 0.1 mM has been demonstrated in the striatum during reperfusion (Hyslop et al., 1995). The severe energy deficit, the complex Na^+ and Ca^{2+} deregulation, and the loss of $\Delta\psi m$ induced by H_2O_2 in Na^+-loaded nerve terminals demonstrated in this study could indicate a mechanism by which cellular injury initiated during ischemia could be further augmented during reperfusion, preventing the restoration of normal cellular functions.

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