

# Plasma Membrane Depolarization and Disturbed $\text{Na}^+$ Homeostasis Induced by the Protonophore Carbonyl Cyanide-*p*-trifluoromethoxyphenyl-hydrazon in Isolated Nerve Terminals

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## ABSTRACT

The effect of the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon (FCCP) was studied on the intracellular  $[\text{Na}^+]_i$ , pH, and plasma membrane potential in isolated nerve terminals. FCCP induced a rise of  $[\text{Na}^+]_i$  at, and even below, the concentrations (0.025–1  $\mu\text{M}$ ) in which it is usually used in intact cells to eliminate  $\text{Ca}^{2+}$  uptake by mitochondria. The FCCP-induced increase of  $[\text{Na}^+]_i$  correlates with a fall in both the ATP level and the ATP/ADP ratio. In addition, a sudden rise of the intracellular proton concentration ( $[\text{H}^+]_i$ ) from  $83 \pm 0.4$  to  $124 \pm 0.7$  nM was observed on the addition of FCCP (1  $\mu\text{M}$ ). Parallel with the rise in  $[\text{H}^+]_i$ , an abrupt depolarization was detected, followed by a slower decrease in the plasma membrane potential. Both the extent of the  $\text{pH}_i$  change and the fast depolarization of the plasma membrane were proportional to the proton

electrochemical gradient across the plasma membrane; when this gradient was increased, greater depolarization was detected. The slower decrease of the membrane potential after the fast initial depolarization was abolished when the medium contained no  $\text{Na}^+$ . It is concluded that FCCP (1) gives rise to a depolarization by setting the plasma membrane potential close to the proton equilibrium potential and (2) enhances the intracellular  $[\text{Na}^+]_i$  as a consequence of an insufficient ATP level and ATP/ADP ratio to fuel the  $\text{Na}^+, \text{K}^+/\text{ATPase}$ . Because both disturbed  $\text{Na}^+$  homeostasis and plasma membrane depolarization could profoundly interfere with  $\text{Ca}^{2+}$  homeostasis in the presence of protonophores, consideration given to these alterations may help to clarify the cellular  $\text{Ca}^{2+}$  sequestration processes.

The primary effect of protonophores is to collapse the proton gradient across the mitochondrial inner membrane, resulting in a complete abolition of the mitochondrial membrane potential and  $\text{Ca}^{2+}$  accumulation (Gunter and Pfeiffer, 1990). For this reason, they have been widely used as tools to study the role of mitochondria in maintaining the physiological  $\text{Ca}^{2+}$  homeostasis of the cells. Protonophores were first exploited in isolated nerve terminals and were used to establish the role of mitochondria in the regulation of  $[\text{Ca}^{2+}]_i$  (Akerman and Nicholls, 1981). Recent studies with protonophores in some neurons (dorsal root ganglion neurons: Thayer and Miller, 1990; Werth and Thayer, 1994; cultured cortical neurons: White and Reynolds, 1995, 1997; cerebellar granule cells: Kiedrowski and Costa, 1995; hippocampal neurons Wang and Thayer, 1996), but not all, (septal neurons: Bleakman *et al.*, 1993) provided evidence for the primary role of mitochondria in regulating the recovery of  $[\text{Ca}^{2+}]_i$  from a

$\text{Ca}^{2+}$  load. In these studies, cells were stimulated either by depolarization with high  $[\text{K}^+]$  (Thayer and Miller, 1990; Werth and Thayer, 1994) or by the excitotoxic neurotransmitter glutamate (Bleakman *et al.*, 1993; White and Reynolds, 1995, 1997; Kiedrowski and Costa, 1995; Wang and Thayer, 1996).

A principal assumption in the use of protonophores is that their major cellular target is mitochondria, with no or negligible effects on the plasma membrane, so alterations of the  $\text{Ca}^{2+}$  transients in the presence of protonophores are assumed to reflect the involvement of mitochondria in shaping the stimulation-evoked  $[\text{Ca}^{2+}]_i$  signals. However, the report that FCCP could mobilize  $\text{Ca}^{2+}$  from nonmitochondrial pools in *Helisoma* neurons indicates that cellular processes other than mitochondrial function also may be influenced (Jensen and Rehder, 1991).

By dissipating the proton gradient, protonophores cause a rapid loss of ATP; not only is ATP synthesis stopped, but also ATP is hydrolyzed by the mitochondrial ATP synthase (Budd and Nicholls, 1996). In theory, glycolysis may compensate, at

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**ABBREVIATIONS:**  $[\text{Ca}^{2+}]_i$ , intracellular  $\text{Ca}^{2+}$  concentration; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon; SBFI,  $\text{Na}^+$ -binding benzofuran isophthalate;  $[\text{Na}^+]_i$ , intracellular  $\text{Na}^+$  concentration; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid;  $\text{pH}_i$ , intracellular pH; BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; JC-1, 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazoly carbocyanine iodide.

least in part, for the loss of the mitochondrial ATP production; however, even in isolated nerve terminals, where the rate of this pathway is increased 10-fold in the presence of protonophores, it is insufficient to maintain the normal ATP/ADP ratio (Kauppinen and Nicholls, 1986). It has been shown that in cerebellar granule cells, the increase in the depolarization-evoked  $Ca^{2+}$  transients by protonophores is a direct consequence of ATP depletion rather than inhibition of the mitochondrial  $Ca^{2+}$  transport (Budd and Nicholls, 1996).

An insufficient ATP level is expected to inhibit many ATP-dependent processes. The  $Na^+$ ,  $K^+$ /ATPase, which has a fundamental importance in maintaining the  $Na^+$  equilibrium across the plasma membrane, may be one of the impaired enzymes, yet this aspect of the action of protonophores has been given surprisingly little attention. It can be predicted that if the decrease in the ATP level and ATP/ADP ratio is sufficiently large to inhibit the  $Na^+$ ,  $K^+$ /ATPase, this would result in an increase of  $[Na^+]_i$  and depolarization of the plasma membrane.

In addition, being protonophores, these agents may also alter the pH gradient across the plasma membrane. This possibility arises from the observation that protonophores induce intracellular acidification in cultured dorsal root ganglion cells (Werth and Thayer, 1994) and hippocampal neurons (Wang *et al.*, 1994, 1995). Given the large electrochemical proton gradient across the plasma membrane, proton influx brought about by protonophores could result in marked plasma membrane depolarization.

In the current study, the effect of the protonophore FCCP was investigated on the  $Na^+$  homeostasis,  $pH_i$ , and plasma membrane potential of isolated nerve terminals prepared from the brain cortex of guinea pigs. We report that FCCP greatly reduces the proton electrochemical gradient of the plasma membrane and induces a large plasma membrane depolarization. In addition, it enhances  $[Na^+]_i$  in parallel with a fall in both ATP level and ATP/ADP ratio. The significance of this study is that both the enhanced  $[Na^+]_i$  and the plasma membrane depolarization could interfere, in a number of ways, with  $Ca^{2+}$  homeostasis; hence consideration of these effects of FCCP may facilitate understanding of the mechanisms by which  $[Ca^{2+}]_i$  is regulated in the cells.

## Materials and Methods

**Preparation of synaptosomes.** Isolated nerve terminals (synaptosomes) were prepared from brain cortex of guinea pigs as detailed previously (Adam-Vizi and Ligeti, 1984). Synaptosomes from an 0.8 M sucrose gradient were diluted with ice-cold distilled water to a concentration of 0.32 M. The pellet obtained after centrifugation at  $20,000 \times g$  for 20 min was suspended in 0.32 M sucrose (20 mg/ml of protein) and kept on ice. For further manipulation, aliquots of synaptosomes were incubated in a standard medium (140 mM NaCl, 3 mM KCl, 2 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , 10 mM PIPES, pH 7.38, 10 mM glucose) as detailed below. All the experiments were carried out at 37°.

**Determination of  $[Na^+]_i$ .** Synaptosomes (2 mg/ml) were incubated in standard medium in which  $Na^+$  had been replaced with an equimolar amount of sucrose, and 10  $\mu$ M SBFI ( $Na^+$ -binding benzofuran isophthalate) acetoxymethyl ester and pluronic acid (0.3%) added, for 60 min at 37°. After sedimentation and washing three times with a  $Na^+$ -free standard medium, the pellet was resuspended in the same medium (8 mg/ml), and 50- $\mu$ l aliquots were used in cuvettes containing 2.0 ml of standard medium. All samples were assayed after preincubation at 37° for 5 min. The fluorescence of

intrasynaptosomally trapped SBFI was measured using 340/380-nm excitation and 510-nm emission wavelengths in a Deltascan fluorescence spectrophotometer (PTI, Monmouth Junction, NJ). A calibration curve was used to quantify  $[Na^+]_i$  (in millimolar). The calibration curve was made in the presence of 3  $\mu$ M gramicidin in a medium containing different concentrations of  $Na^+$ ; details of the method have been described previously (Deri and Adam-Vizi, 1993). The fluorescent signal of SBFI was insensitive to changes in pH between 7.5 and 6.7. pH was changed in this range in SBFI-loaded synaptosomes previously treated with gramicidin (2.5  $\mu$ M), monensin (8  $\mu$ M), and nigericin (8  $\mu$ M), which equilibrated the external and internal  $Na^+$  and  $H^+$  concentrations (see below), and no significant change in the fluorescence of the dye was observed.

**ATP and ADP measurement.** Incubation of synaptosomes was carried out at 37° in the standard medium in the absence or presence of FCCP as indicated. 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 Luminometer 1251 (Turku, Finland). Results are expressed as nmol of ATP/mg of synaptosomal protein and as ATP/ADP ratio.

**Measurement of  $pH_i$ .**  $pH_i$  of synaptosomes was measured as described previously (Nachshen and Drapeau, 1988). Synaptosomes (4 mg/ml) were loaded with the acetoxymethyl ester of BCECF present in the standard medium (5  $\mu$ M) for 20 min at 37° and then washed three times. Synaptosomes were resuspended in the standard medium (8 mg/ml) and kept on ice. For measurement of fluorescence, 50- $\mu$ l aliquots of synaptosomes were diluted in 2 ml of medium. Fluorescence ratios were determined in a PTI Deltascan spectrofluorometer using 440- and 505-nm excitation and 540-nm emission wavelengths. Leakage of BCECF from synaptosomes was determined by adding unloaded synaptosomes to the supernatant of BCECF-loaded synaptosomes. Corrections were made after each measurement by subtracting the fluorescence of the leaked BCECF from those of the experimental values. For a calibration curve (created for each experiment), the external and internal  $[H^+]$  values were equilibrated at varying extracellular pH values by the addition of 8  $\mu$ M nigericin ( $K^+$ / $H^+$  antiporter), 2.5  $\mu$ M gramicidin ( $Na^+$ / $K^+$  ionophore), and 8  $\mu$ M monensin ( $Na^+$ / $H^+$  antiporter).

**Determination of plasma membrane potential.** Plasma membrane potential of synaptosomes was measured by the cationic fluorescent probe 3,3'-diethylthiocarbocyanine iodide as described previously (Hare and Atchison, 1992). Synaptosomes (0.5 mg/ml) were preincubated in the standard medium with 0.5  $\mu$ M dye for 5 min in the presence of 2  $\mu$ M rotenone and 10  $\mu$ M oligomycin, which depolarize mitochondria without affecting plasma membrane potential (Scott and Nicholls, 1980). The fluorescence of the dye was monitored in a Perkin Elmer LS-50 fluorescence spectrometer using 565-nm excitation and 584-nm emission wavelengths. Calibration of membrane potential was based on the earlier observation that a linear relationship exists between dye fluorescence and plasma membrane potential (Hare and Atchison, 1992).

**Determination of mitochondrial membrane potential.** JC-1 is a membrane potential sensitive probe which accumulates in energized mitochondria and subsequently forms J-aggregates from monomers. J-aggregate formation, which can be followed by the fluorescence change at 590 nm where it exhibits an emission maximum, has been suggested to be the function of the membrane potential of the mitochondria (Reers *et al.*, 1991). Synaptosomes were suspended in  $Ca^{2+}$ -free standard medium (4 mg/ml) and loaded with JC-1 (10  $\mu$ g/ml) for 15 min at 37°. After sedimentation synaptosomes were washed twice, resuspended in standard medium (8 mg/ml) and kept on ice. For measurement of fluorescence 50  $\mu$ l aliquots were diluted in 2 ml standard medium at 37°. Fluorescence intensity was determined in a PTI Deltascan fluorescence spectrophotometer at 535 and 595 emission wavelength (with excitation at 490 nm) corresponding to the fluorescence peak of the monomer and that of the aggregate, respectively (Reers *et al.*, 1991).

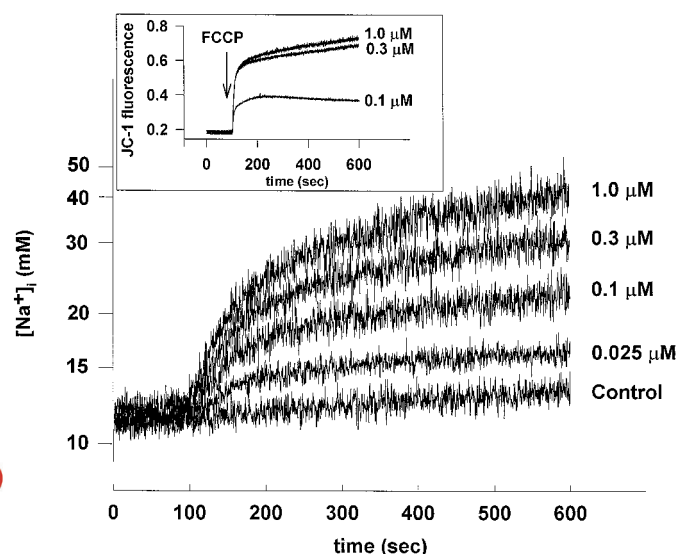
**Materials.** Standard laboratory chemicals were obtained from Sigma Chemical (St. Louis, MO). Fura-2, 3,3'-diethylthiocarbocyanine iodide, and SBFI were purchased from Calbiochem (San Diego, CA). JC-1 was obtained from Molecular Probes (Eugene, OR).

**Statistics.** Results are expressed as mean  $\pm$  standard error values. For statistical analysis, Student's *t* test was used. Differences were considered significant at a level of  $p < 0.05$ .

## Results

**Increase of  $[\text{Na}^+]_i$  elicited by FCCP.**  $[\text{Na}^+]_i$  concentration in synaptosomes as detected by the fluorescence of the SBFI was found to be  $12.5 \pm 3$  mM, in agreement with our previous reports on this preparation (Deri and Adam-Vizi, 1993; Tretter and Adam-Vizi, 1996). Fig. 1 shows that on the addition of FCCP, an increase in the  $[\text{Na}^+]_i$  was detected. The effect was concentration dependent, with  $0.025 \mu\text{M}$  FCCP enhancing  $[\text{Na}^+]_i$  by a 2–3 mM and  $1 \mu\text{M}$  FCCP increasing the  $[\text{Na}^+]_i$  to  $42 \pm 4$  mM over an incubation period of 8 min. After a relatively rapid increase in the first 2 min,  $[\text{Na}^+]_i$  increased only slowly during further incubation with FCCP. Fig. 1 (*inset*) shows that FCCP in this concentration range, as expected, reduces membrane potential of mitochondria occluded within the nerve terminals. FCCP at  $1 \mu\text{M}$  seems to be sufficient to collapse mitochondrial membrane potential; with a higher FCCP concentration, no further change in JC-1 fluorescence could be elicited.

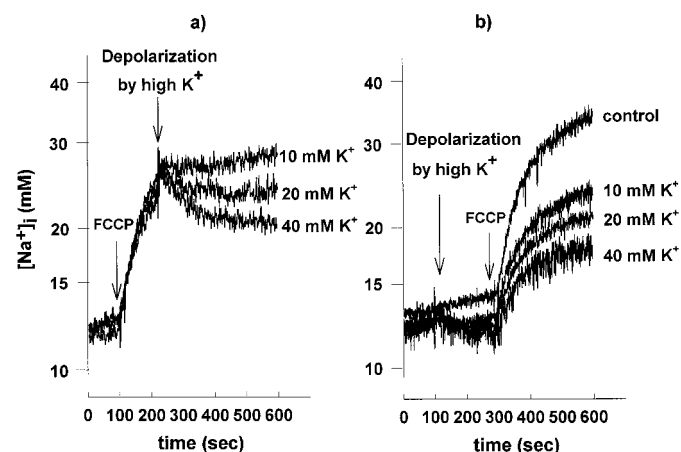
The  $[\text{Na}^+]_i$  rise in response to FCCP was independent of the presence of external  $\text{Ca}^{2+}$ ; the extent of the increase of  $[\text{Na}^+]_i$  was the same in the absence of  $\text{Ca}^{2+}$  (no added  $\text{Ca}^{2+}$  and  $1 \text{ mM}$  EGTA in the medium) (not shown), indicating that this effect is not likely to be related to the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger present in the plasma membrane of the nerve terminals (Gill et al., 1981). Preincubation with  $1 \mu\text{M}$  tetrodotoxin did not prevent the FCCP-induced increase of  $[\text{Na}^+]_i$  (data not shown).



**Fig. 1.**  $[\text{Na}^+]_i$  rise in response to FCCP.  $[\text{Na}^+]_i$  was determined according to the fluorescence of SBFI as described in the text. FCCP at the concentrations indicated was added at 100 sec. *Traces*, representative of eight experiments performed with three different preparations. *Inset*, ratio fluorescence (535/595 nm) of JC-1, the mitochondrial membrane potential sensitive probe due to FCCP. *Traces*, representative of six experiments performed with three different preparations.

**FCCP-evoked  $[\text{Na}^+]_i$  rise results from  $\text{Na}^+$  entry driven by the  $\text{Na}^+$  electrochemical gradient.** The permeability of the plasma membrane to  $\text{Na}^+$  is very limited, allowing a slow entry of  $\text{Na}^+$  down the  $\text{Na}^+$  electrochemical gradient, which is normally balanced by  $\text{Na}^+$  extrusion via the  $\text{Na}^+/\text{K}^+/\text{ATPase}$ . If this balance is disturbed, the concentration of  $\text{Na}^+$  in the cytoplasm is expected to increase. Fig. 2 shows that the increase of  $[\text{Na}^+]_i$  in response to FCCP is the result of  $\text{Na}^+$  entry driven by the  $\text{Na}^+$  electrochemical gradient across the plasma membrane. After an incubation for 2 min with  $1 \mu\text{M}$  FCCP,  $\text{K}^+$  was added in high concentrations to impose a clamped depolarization, hence reducing the  $\text{Na}^+$  electrochemical gradient. The increase of  $[\text{Na}^+]_i$  elicited by FCCP was immediately halted on the addition of  $\text{K}^+$  (Fig. 2a). In the presence of  $10 \text{ mM}$   $\text{K}^+$ , a new equilibrium at  $27 \pm 3 \text{ mM}$   $[\text{Na}^+]_i$  seemed to be maintained, and at a higher  $[\text{K}^+]$ ,  $[\text{Na}^+]_i$  not only stopped rising but actually began to fall slowly. This may indicate that at larger depolarizations when the  $\text{Na}^+$  entry is reduced due to the reduced  $\text{Na}^+$  electrochemical gradient, the activity of the  $\text{Na}^+/\text{K}^+/\text{ATPase}$  is sufficient to extrude a considerable part of the  $\text{Na}^+$  that has accumulated in the cytoplasm. Similar results were obtained when we applied the high  $\text{K}^+$  depolarization in the absence of external  $\text{Ca}^{2+}$  ( $1 \text{ mM}$  EGTA was present), ruling out the possibility that in greatly depolarized synaptosomes, the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger working in the reverse mode ( $\text{Ca}^{2+}$  in,  $\text{Na}^+$  out) could contribute to the  $\text{Na}^+$  extrusion. The same conclusion can be drawn with regard to the  $\text{Na}^+$  electrochemical gradient from experiments in which FCCP was applied in synaptosomes previously depolarized by high  $[\text{K}^+]$  (Fig. 2b). In predepolarized synaptosomes, FCCP elicited a smaller increase in  $[\text{Na}^+]_i$ , and the reduction observed in the effect of FCCP seemed to be proportional to the increase in  $[\text{K}^+]$  (i.e., with the reduction in the  $\text{Na}^+$  electrochemical gradient).

The possibility that  $\text{K}^+$  at high concentration could acti-



**Fig. 2.** Depolarization produced by high  $[\text{K}^+]$  reduces  $[\text{Na}^+]_i$  rise due to FCCP. **a**, Rise in  $[\text{Na}^+]_i$  due to  $1 \mu\text{M}$  FCCP was stopped by the addition of KCl at the indicated concentrations 2 min after the FCCP application. *Traces*, representative of three independent experiments made in duplicate. Osmolarity of the medium was increased by the addition of KCl; however, this made no contribution to the effect of high  $[\text{K}^+]$  as determined in separate experiments in which osmolarity was increased to the same extent by the addition of sucrose instead of KCl. **b**, Synaptosomes were depolarized by KCl at the indicated concentrations; 3 min later,  $1 \mu\text{M}$  FCCP was added. *Control trace*,  $[\text{Na}^+]_i$  rise in response to FCCP without prior depolarization (no  $\text{K}^+$  added). *Traces*, representative of three independent experiments performed in duplicate.



vate the  $\text{Na}^+, \text{K}^+/\text{ATPase}$ , accounting for a decrease in  $[\text{Na}^+]_i$ , could be eliminated by considering that the  $K_m$  value of the enzyme for  $\text{K}^+$  is  $\sim 0.5$  mM (Erecinska *et al.*, 1996), which means that 3 mM  $\text{K}^+$  present in our standard medium should be near the saturating concentration, and an increase above 10 mM should not greatly activate the enzyme.

**The effect of FCCP on  $[\text{Na}^+]_i$  is paralleled by a reduction in the ATP/ADP ratio.** It has been shown that protonophores decrease the ATP content and  $[\text{ATP}]/[\text{ADP}]$  ratio in neuronal preparations (Kauppinen and Nicholls, 1986; Budd and Nicholls, 1996). In those investigations, high concentrations of protonophores were generally used. We investigated the effect of FCCP on the ATP level and  $[\text{ATP}]/[\text{ADP}]$  ratio using the same concentrations as those for the study of  $[\text{Na}^+]_i$  (Fig. 1). Fig. 3 shows that the ATP level of the synaptosomes was decreased by FCCP and that the effect was proportional to the concentration used. The maximal effect of FCCP was achieved within 2 min, and longer incubations caused no further change in ATP level or  $[\text{ATP}]/[\text{ADP}]$  ratio (not shown). Because of the limitations of the method for determining the ATP and ADP content, reliable data cannot be obtained with incubation periods of  $< 2$  min. It seems that the effect of FCCP on  $[\text{Na}^+]_i$  is paralleled by a reduction in the ATP level and ATP/ADP ratio. The only inconsistency is that on increasing the concentration of FCCP from 0.3 to 1  $\mu\text{M}$ , a larger increase in  $[\text{Na}^+]_i$  is seen, whereas ATP level is not reduced further.

It was considered whether  $[\text{Na}^+]_i$  rise itself could contribute to the decrease in the ATP level due to a greater utilization of ATP by the activated  $\text{Na}^+, \text{K}^+/\text{ATPase}$  of the plasma membrane. However, reduction in the ATP level in response to FCCP was not different in synaptosomes pretreated with 1 mM ouabain for 5 min (not shown), arguing against this possibility.

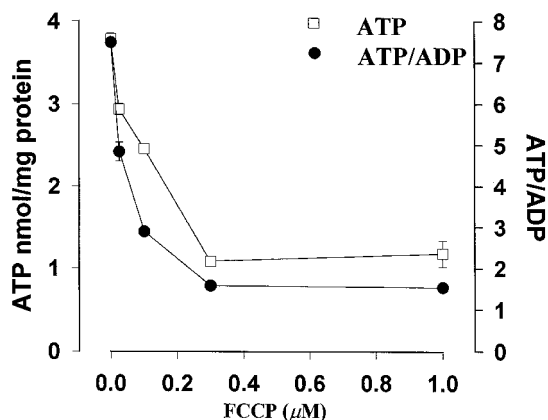
**Depolarization of the plasma membrane by FCCP.** An increase in  $[\text{Na}^+]_i$ , if sufficiently large, is expected to result in a depolarization of the nerve terminals. The plasma membrane potential at different  $[\text{Na}^+]_i$  values can be calculated from the constant field equation with the assumption that the entry of  $\text{Na}^+$  is followed by the efflux of an equimolar amount of  $\text{K}^+$ . According to this calculation, the resting

membrane potential of  $\sim -70$  mV is expected to decrease by 9 mV when the  $[\text{Na}^+]_i$  is raised from 12 to 42 mM, a condition brought about by the presence of 1  $\mu\text{M}$  FCCP for 8 min (Fig. 1). This prediction was tested by following the fluorescence change of a membrane potential-sensitive dye (3,3'-diethylthiocarbocyanine iodide) in synaptosomes treated with FCCP. Because the dye equilibrates not only in the cytosol but also in the mitochondria, plasma membrane potential can be measured only when the mitochondrial membrane potential has been eliminated previously. This can be accomplished by the simultaneous presence of rotenone (2  $\mu\text{M}$ ) and oligomycin (10  $\mu\text{M}$ ), which leaves the plasma membrane potential unaltered (Scott and Nicholls, 1980). Fig. 4a shows that in the presence of these mitochondrial inhibitors, the resting plasma membrane potential can be monitored and the depolarizing effect of 20 mM  $\text{K}^+$  can be demonstrated (Fig. 4a).

The resting membrane potential calculated in 35 independent experiments was  $-77 \pm 4$  mV. On the addition of FCCP to synaptosomes previously treated with rotenone/oligomycin, a fall in the membrane potential was seen (Fig. 4b). The effect of FCCP had a fast and a slower component: the initial fast component was characterized by a depolarization produced within 20 sec after the FCCP addition, and this was followed by a slower, additional decrease of the plasma membrane potential over an incubation period of 5 min. The initial fast depolarization by 1  $\mu\text{M}$  FCCP was  $34 \pm 3$  mV, far greater than that expected from the increase of  $[\text{Na}^+]_i$  according to the Goldman-Hodgkin-Katz equation. In addition, it is too fast to be the result of  $\text{Na}^+$  entry; as shown in Fig. 1, the FCCP-elicited rise of  $[\text{Na}^+]_i$  is much slower, resulting in no more than a 5–6 mM increase in  $[\text{Na}^+]_i$  within the first 20 sec.

To establish the contribution of  $\text{Na}^+$ -entry to the FCCP-evoked depolarization, synaptosomes were incubated in a  $\text{Na}^+$ -free medium ( $\text{Na}^+$  was replaced by an equimolar amount of *N*-methylglucamine) and then challenged with 1  $\mu\text{M}$  FCCP. Fig. 4c shows that this did not prevent the initial rapid depolarization elicited by FCCP, indicating that the depolarization is unrelated to  $\text{Na}^+$  entry; however, the subsequent slow decrease in the membrane potential was eliminated, suggesting that this may be the result of the rise in  $[\text{Na}^+]_i$ .

**Decreased  $\text{pH}_i$  in response to FCCP.** To reveal the underlying mechanism of the sudden,  $\text{Na}^+$ -independent depolarization observed on the addition of FCCP,  $\text{pH}_i$  was followed under the same conditions. At an inside negative resting membrane potential of  $\sim -77$  mV and external  $[\text{H}^+]$  of 41 nM ( $\text{pH}$  7.38), the  $\text{pH}_i$  should be  $\sim 6.13$  (730 nM  $[\text{H}^+]_i$ ) to ensure an equilibrium distribution of  $\text{H}^+$  across the plasma membrane. In fact, the proton distribution is far from this equilibrium, with a  $\text{pH}_i$  of  $7.08 \pm 0.031$ , which is maintained by the tonic activity of the  $\text{Na}^+/\text{H}^+$  exchanger (Sauvaigo *et al.*, 1984; Nachsen and Drapeau, 1988). It follows that at this small ( $83 \pm 0.4$  nM)  $[\text{H}^+]_i$ , there is a large electrochemical proton gradient at the plasma membrane that, in the presence of FCCP, could be the driving force for  $\text{H}^+$  entry across the plasma membrane. Wang *et al.* (1995) suggested that high concentration of protonophores equilibrates  $\text{H}^+$  across the plasmalemma in a  $\text{pH}$ - and voltage-dependent manner. Fig. 5 shows that, indeed, FCCP elicits an immediate drop in the  $\text{pH}_i$ , reaching its maximum within 20 sec.  $\Delta\text{pH}_i$  due to 1



**Fig. 3.** Effect of FCCP on ATP level (nmol/mg of protein) and ATP/ADP ratio in the presence of 0.025–1  $\mu\text{M}$  FCCP. Synaptosomes were incubated in the presence of FCCP for 2 min, and ATP and ADP levels were determined. Points, average of four determinations made in duplicate  $\pm$  standard error (not indicated where it is smaller than the symbols). The difference at each point is statistically significant ( $p < 0.01$ ) compared with the controls.

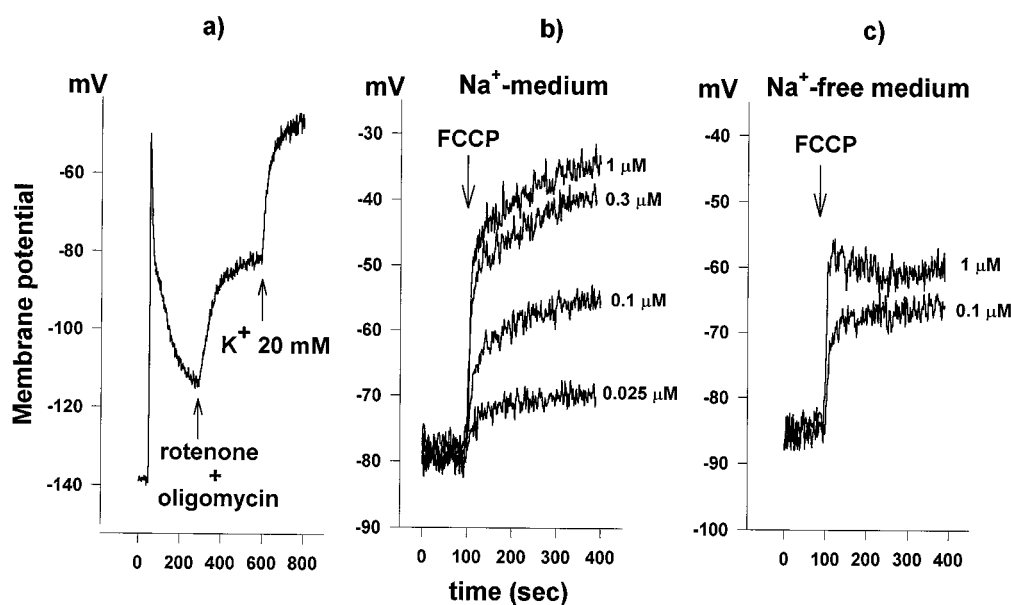
$\mu\text{M}$  FCCP is  $0.175 \pm 0.014$  ( $\text{pH}_i$  is decreased from  $7.08 \pm 0.031$  to  $6.905 \pm 0.035$ ,  $p < 0.05$ , five experiments). With a  $\text{pH}_i$  of 6.9, the new  $\text{H}^+$  distribution across the plasma membrane brought about by  $1 \mu\text{M}$  FCCP would be in equilibrium at a plasma membrane potential of  $\sim -29 \text{ mV}$ , as calculated with the Nernst equation. It is shown in Fig. 4b that we detected a more negative membrane potential in the presence of FCCP (considering only the  $\text{Na}^+$ -independent fast depolarization), which indicates that FCCP greatly reduces but does not completely eliminate the proton electrochemical gradient of the plasma membrane.

Theoretically, if FCCP elicits a rise of  $[\text{Ca}^{2+}]_i$ , as shown in a number of studies (Duchen et al., 1990; Bleakman et al., 1993; White and Reynolds, 1995, 1997), then this may also induce local acidification at the inner surface of the plasma membrane by displacing  $\text{H}^+$  from intracellular sites (Meech and Thomas, 1977). In our experiments, this is unlikely to be a contributing factor because  $\text{pH}_i$  change due to FCCP was not different in a  $\text{Ca}^{2+}$ -free medium, in which FCCP fails to enhance  $[\text{Ca}^{2+}]_i$  in isolated nerve terminals (Tretter L and Adam-Vizi V, unpublished observation).

**Correlation between  $\Delta\text{pH}_i$  and the depolarization due to FCCP.** From the experiments presented, it seems very likely that the FCCP-elicited plasma membrane depolarization is the result of a proton influx down the electrochemical gradient. To test directly this hypothesis, we followed the membrane potential change due to FCCP at different proton electrochemical gradients. Fig. 6 shows that on increasing the  $[\text{H}^+]$  in the medium to  $560 \text{ nM}$  ( $\text{pH}$  is reduced to 6.25), the intracellular proton concentration is increased to  $398 \pm 3 \text{ nM}$  ( $\text{pH}_i$   $6.4 \pm 0.039$ ); however, the  $\text{H}^+$  equilibrium potential under this condition ( $+9.2 \text{ mV}$ ) is even farther from the resting membrane potential than at  $\text{pH}_o$  7.38. Thus, the proton electrochemical gradient is larger.

This is, indeed, reflected in a larger intracellular acidification produced by  $1 \mu\text{M}$  FCCP compared with that with  $\text{pH}$  7.38 in the medium (Fig. 6, c and b; Table 1). In contrast, with a  $[\text{H}^+]$  of  $7.4 \text{ nM}$  ( $\text{pH}_o$  8.13) in the medium, the  $[\text{H}^+]_i$  also decreased ( $35.2 \pm 0.2 \text{ nM}$ ;  $\text{pH}_i$  7.45), thus coming close to an equilibrium distribution ( $\text{H}^+$  equilibrium potential in this condition is  $-41 \text{ mV}$ ). In compliance with the greatly reduced proton electrochemical gradient, FCCP produced only a slight intracellular acidification, which seems to be rapidly regulated. The effect of FCCP was consistent in each experiment ( $\Delta\text{pH}_i$   $0.101 \pm 0.011$  measured 20 sec after the addition of FCCP), although the difference in  $\text{pH}_i$  values measured before and after FCCP addition was not statistically significant (Table 1).

By following the plasma membrane potential under these conditions, a remarkable quantitative correlation was found between the  $[\text{H}^+]_i$  change and the depolarization in response to FCCP. It is clear from the results shown in Fig. 6 that with a larger proton electrochemical gradient at the plasma membrane, FCCP elicits a greater depolarization, and as the gradient is decreased, the extent of depolarization due to FCCP also is reduced. Given the assumption that FCCP, as a protonophore, completely collapses the proton gradient of the plasma membrane, the plasma membrane potential in the presence of FCCP should be equal to the  $\text{H}^+$  equilibrium potential. Although FCCP depolarized the membrane to an extent close to this predicted value, the membrane potential at each condition was found to be more negative than the  $\text{H}^+$  equilibrium potential. This is shown in Table 1, which gives a quantitative summary of the results presented in Fig. 6 (columns 1, 2, and 4) and the calculated proton equilibrium potential in the presence of  $1 \mu\text{M}$  FCCP (column 5). These data corroborate the suggestion that FCCP is able to dimin-



**Fig. 4.** Effect of FCCP on the plasma membrane potential. Membrane potential was determined according to the fluorescence method described in the text. a, Representative recording showing the fluorescence change on the addition of synaptosomes, rotenone plus oligomycin and high  $[\text{K}^+]$ , respectively. Synaptosomes added at 50 sec decreased the fluorescence, presumably as a consequence of dye accumulation in the synaptosomal membranes. Oligomycin ( $10 \mu\text{M}$ ) and rotenone ( $2 \mu\text{M}$ ) were added at 300 sec to collapse mitochondrial membrane potential. To depolarize the plasma membrane,  $20 \text{ mM}$   $\text{K}^+$  was added as indicated. Increase in the fluorescence indicates depolarization. b, FCCP at the indicated concentrations was added to synaptosomes previously incubated for 5 min in the presence of rotenone ( $2 \mu\text{M}$ ) and oligomycin ( $10 \mu\text{M}$ ). c, Synaptosomes were preincubated as for b except that  $\text{Na}^+$  in the medium was replaced by equimolar  $N$ -methylglucamine. Traces, representative of three determinations. In b and c, plasma membrane potential is calculated in mV.

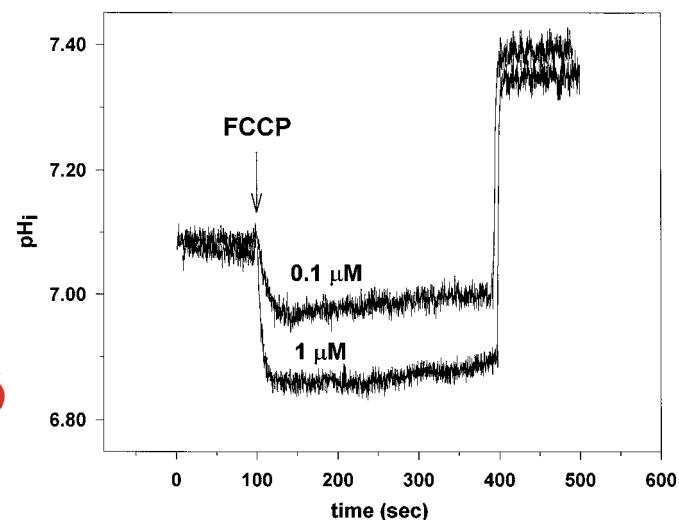
ish but not totally abolish the proton electrochemical gradient of the plasma membrane.

The correlation between the plasma membrane depolarization and the increase of the intracellular proton concentration supports the hypothesis that the FCCP-induced depolarization of the plasma membrane is the consequence of  $H^+$  influx into the nerve terminals down the proton electrochemical gradient.

## Discussion

The major observation of this study was that FCCP in, and even below, the concentrations generally used in intact cells to manipulate the  $Ca^{2+}$  sequestration of the mitochondria gives rise to (1) depolarization of the plasma membrane and (2) an increase in the  $[Na^+]_i$  in isolated nerve terminals. The importance of these observations is that both the elevated  $[Na^+]_i$  and the plasma membrane depolarization may profoundly influence  $Ca^{2+}$  homeostasis, which should be considered when experiments with FCCP are designed. These effects also were produced by another protonophore, carbonyl cyanide *m*-chlorophenylhydrazone, which was applied in a few control experiments (data not shown).

**Plasma membrane depolarization.** In accordance with previous observations made in cultured dorsal root ganglion cells (Werth and Thayer, 1994) and hippocampal neurons (Wang *et al.*, 1994, 1995), we detected an intracellular acidification in isolated nerve terminals in response to FCCP (Figs. 5 and 6). The increase of the intracellular proton concentration seems to be correlated with the proton electrochemical gradient of the plasma membrane and is likely to be the result of proton entry via the plasma membrane, which is consistent with the suggestion made by Wang *et al.* (1995). The proton electrochemical gradient is not completely collapsed by FCCP; at the membrane potential measured in the presence of FCCP and with the increased  $[H^+]_i$ , the proton electrochemical gradient of the plasma membrane is greatly reduced but not completely eliminated (Table 1). Although this seems to be the major mechanism of the  $pH_i$  change, it

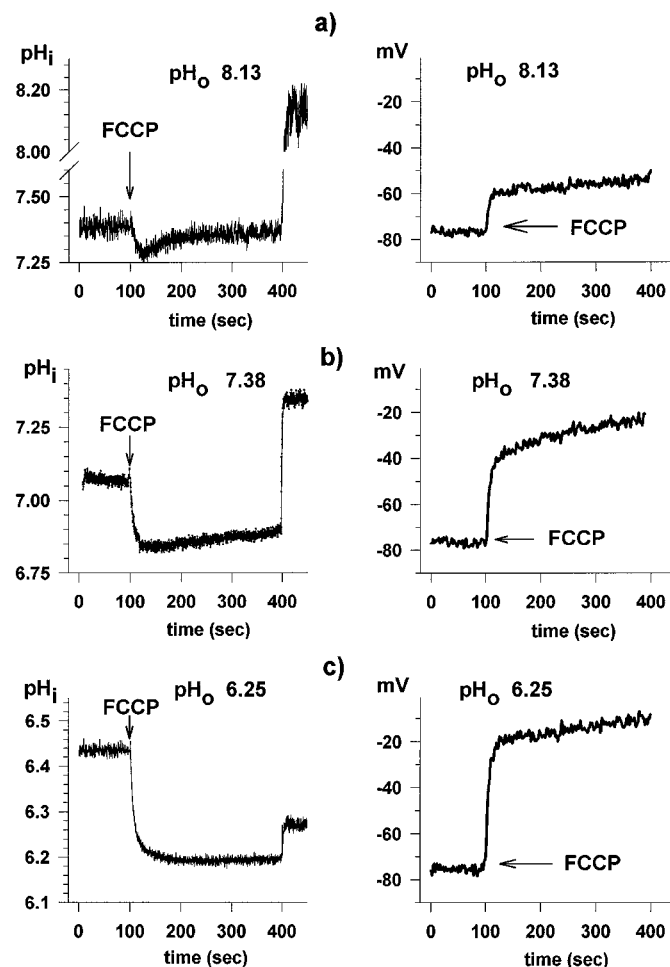


**Fig. 5.** Effect of FCCP at two different concentrations on synaptosomal  $pH_i$ . FCCP (1.0 or 0.1  $\mu M$ ) was added to synaptosomes loaded with BCECF as indicated. At 400 sec, nigericin (8  $\mu M$ ), monensin (8  $\mu M$ ), and gramicidin (2.5  $\mu M$ ) were added to equilibrate the extracellular and intracellular pH. Traces, representative of five experiments.

cannot be excluded that protons also may be released by FCCP from the synaptic vesicles within the axon terminals, which have a highly acidic interior.

It is uncertain whether the relatively small decrease of  $pH_i$  brought about by FCCP itself could have an impact on the  $Ca^{2+}$ -sequestering processes. In synaptosomes, cytoplasmic acidification does not itself seem to influence significantly  $[Ca^{2+}]_i$  (Drapeau and Nachshen, 1988); however, in cultured hippocampal neurons, a delayed recovery of  $[Ca^{2+}]_i$  after glutamate stimulation was observed when the  $pH_i$  was reduced to 6.6 (Koch and Barish, 1994).

Partial dissipation of the proton gradient by FCCP, while inducing only a slight intracellular acidification, is accompanied by a remarkable decrease of the plasma membrane potential. In the presence of FCCP (1  $\mu M$ ), the plasma membrane potential measured in this study seems to be close to the estimated proton equilibrium potential (Table 1). The extent of depolarization induced by 1  $\mu M$  FCCP ( $34 \pm 3$  mV) is far greater than that sufficient to open voltage-dependent  $Ca^{2+}$  channels of the plasma membrane. In synaptosomes, the threshold depolarization for a voltage-dependent  $Ca^{2+}$  entry was found to be 10–12 mV (Adam-Vizi and Ligeti, 1986); therefore, FCCP is assumed to induce  $Ca^{2+}$  influx into



**Fig. 6.**  $pH_i$  (left), and plasma membrane potential (mV, right) measured in parallel samples at external pH ( $pH_o$ ) of 8.13 (a), 7.38 (b), or 6.25 (c). FCCP at 1  $\mu M$  was added at 100 sec; then,  $pH_i$  was equilibrated at 400 sec by the addition of nigericin (8  $\mu M$ ), monensin (8  $\mu M$ ) and gramicidin (2.5  $\mu M$ ). Traces, representative of four (a and c) or five (b) experiments.



the terminals. This seems to happen in cultured cortical neurons, in which the  $[Ca^{2+}]_i$  rise evoked by 5  $\mu M$  FCCP could be attenuated by nimodipine and  $\omega$ -conotoxin, blockers of the L- and N-type  $Ca^{2+}$  channels, respectively (White and Reynolds, 1995). Thus, in the interpretation of the FCCP-evoked  $[Ca^{2+}]_i$  signal in different preparations, it is important to realize that besides mobilizing the mitochondrial  $Ca^{2+}$  pool, protonophores may provoke  $Ca^{2+}$  influx as a result of a plasma membrane depolarization.

It should be noted that no protonophore-elicited depolarization was detected when  $^{86}Rb$  distribution was followed in synaptosomes (Scott and Nicholls, 1980). The reason for this discrepancy may arise from the very limited time resolution of that method. Even with high  $[K^+]_i$ , which elicits an instantaneous depolarization, 10 min is necessary for  $Rb^+$  to equilibrate across the plasma membrane (Scott and Nicholls, 1980; Adam-Vizi and Ligeti, 1984). In the study of Scott and Nicholls (1980), the preparation was incubated with FCCP for only  $\sim 5$  min, which may not be sufficient for the nerve terminals to attain a new  $^{86}Rb$  equilibrium.

Consistent with our observations are the results obtained in a patch-clamp study of glial cells, in which 1  $\mu M$  FCCP elicited an inward current giving rise to depolarization by 34 mV (Brismar and Collins, 1993). The nature of the current was not characterized, but it was unrelated to the fall in the cellular  $[ATP]$  or changes in the  $[Ca^{2+}]_i$  and indicated a direct effect of FCCP on the plasma membrane. It is possible that the current they obtained was produced by proton influx.

**Disturbed  $Na^+$  homeostasis.** Many transport processes in the plasma membrane are coupled to  $Na^+$  entry down the  $Na^+$  electrochemical gradient. The  $Na^+/Ca^{2+}$  exchanger that is present predominantly in the plasma membrane of the nerve terminals (Gill *et al.*, 1981) plays a major role in maintaining the resting  $[Ca^{2+}]_i$  and restoring  $[Ca^{2+}]_i$  after a  $Ca^{2+}$  load (Sanchez-Armass and Blaustein, 1987). The importance of this system in buffering  $[Ca^{2+}]_i$  after a stimulation-evoked  $Ca^{2+}$  load also has been demonstrated in pituitary cell lines (Korn and Horn, 1989), cultured hippocampal neurons (Segal and Manor, 1992; Koch and Barish, 1994), cerebellar granule cells (Kiedrowski *et al.*, 1994), and cortical neurons (White and Reynolds, 1995). When the  $[Na^+]_i$  increases,  $Ca^{2+}$  efflux mediated by the  $Na^+/Ca^{2+}$  exchanger is expected to be reduced, and in parallel with the decrease of the  $Na^+$  electrochemical gradient, an increasing proportion of the exchanger

would be working in the reverse mode, resulting in an  $Ca^{2+}$  influx instead of a  $Ca^{2+}$  extrusion. In cells in which the  $Na^+/Ca^{2+}$  exchanger is of a great importance in regulating  $[Ca^{2+}]_i$ , the impaired  $Ca^{2+}$  sequestration due to FCCP may result not only from its effect on the mitochondria but also from a reduced function of the  $Na^+/Ca^{2+}$  exchanger produced by a  $[Na^+]_i$  increase.

The elevated  $[Na^+]_i$  brought about by FCCP also might be a factor in the effect of the protonophore on the mitochondria.  $Ca^{2+}$  efflux from the mitochondria is known to be mediated by a  $Na^+/Ca^{2+}$  exchanger (Gunter and Pfeiffer, 1990). Very recently, special attention was given to the role of the mitochondrial  $Na^+/Ca^{2+}$  exchange in sequestration of the glutamate-evoked  $Ca^{2+}$  load in cultured forebrain neurons (White and Reynolds, 1997). The mitochondrial  $Na^+/Ca^{2+}$  exchange inhibitor CGP 37157 prevented the FCCP-induced increase of  $[Ca^{2+}]_i$ , and in  $Na^+$ -depleted neurons, FCCP was no longer able to release  $Ca^{2+}$  from the mitochondria (White and Reynolds, 1997). These data together with the current demonstration that FCCP elicits a  $[Na^+]_i$  rise suggest that  $Ca^{2+}$  efflux from the mitochondria in response to FCCP could be mediated, at least in part, by the mitochondrial  $Na^+/Ca^{2+}$  exchange.

Increase in  $[Na^+]_i$  by FCCP was paralleled by a fall in ATP within the nerve terminals and could be interpreted as the result of inhibition of the  $Na^+, K^+/ATPase$ . When the level of ATP is decreased, there not only is an insufficient amount of substrate but also a shift in the pH optimum of the enzyme to more alkaline pH (Skou, 1979). Therefore, intracellular acidification produced by FCCP could contribute to the inhibition of the  $Na^+, K^+/ATPase$  by providing an additional highly unfavorable condition for its function. This might explain why FCCP at 1  $\mu M$  gives rise to a greater increase in  $[Na^+]_i$  than at 0.3  $\mu M$  (Fig. 1), despite the decrease of ATP to the same low level (Fig. 3).

$Na^+/H^+$  exchange also could be a candidate for the mechanism of the  $Na^+$  influx produced by FCCP because the exchanger is known to be activated by intracellular acidification. However, this seems to be unlikely because change in the  $pH_i$  due to FCCP is relatively small, and in this  $pH_i$  range (7.08–6.9),  $Na^+/H^+$  exchanger of the plasma membrane is only slightly activated (Aronson *et al.*, 1982). In addition, the reduced ATP level due to FCCP is expected to decrease the affinity of the exchanger to  $[H^+]_i$  (Orlowski and Grinstein, 1997). Consistent with these observations,  $[Na^+]_i$  rise due to 1  $\mu M$  FCCP was not significantly altered by the amiloride analogue 5-(*N*-ethyl-*N*-isopropyl)amiloride, an inhibitor of the  $Na^+/H^+$  exchanger (not shown).

An important conclusion emerges from analysis of the correlation between the fall of the ATP level and the increase in  $[Na^+]_i$ . It seems that the  $Na^+, K^+/ATPase$  is not completely inhibited even when the ATP level and ATP/ADP ratio is decreased to  $\sim 25\%$  of the control value by 1  $\mu M$  FCCP (Fig. 3). The limited function of the  $Na^+, K^+/ATPase$  in this condition is insufficient to balance  $Na^+$  entry down the electrochemical gradient; thus,  $[Na^+]_i$  is increased, but it is still adequate to extrude  $Na^+$  when the  $Na^+$  electrochemical gradient is greatly reduced, so further entry of  $Na^+$  is limited by high  $K^+$  depolarization (Fig. 2).

The extent and time dependence of the increase of  $[Na^+]_i$  due to FCCP may vary in different preparations and may depend on the concentration of and the exposure time to

TABLE 1

Effect of FCCP on  $pH_i$  and plasma membrane potential ( $\Psi_p$ ) at different  $pH_o$

$pH$  values and the plasma membrane potential were determined in the experiments presented in Fig. 6. 1,  $pH_i$  detected before FCCP was added; 2,  $pH_i$  obtained 20 sec after the addition of 1  $\mu M$  FCCP; 3, resting plasma membrane potential was not influenced by changing the  $pH_o$  to 8.13 or 6.25, so for the calculation, we used all the data obtained at different  $pH_o$  (35 samples); 4, plasma membrane potential 20 sec after the addition of 1  $\mu M$  FCCP (independent of  $Na^+$  entry, see Fig. 4, b and c); and 5,  $H^+$  equilibrium potential ( $\Psi_H$ ) calculated by taking into account the intracellular  $H^+$  concentrations obtained 20 sec after the addition of 1  $\mu M$  FCCP (column 2). Data are the average values of four independent determinations  $\pm$  standard error.

$pH_o$	1: $pH_i$	2: $pH_i$ FCCP	3: $\Psi_p$ resting	4: $\Psi_p$ FCCP	5: $\Psi_H$
					mV
8.13	7.45 $\pm$ 0.048	7.35 $\pm$ 0.049	-77 $\pm$ 4	-62 $\pm$ 2 <sup>a</sup>	-47
7.38	7.08 $\pm$ 0.031	6.905 $\pm$ 0.035 <sup>a</sup>	-77 $\pm$ 4	-43 $\pm$ 3 <sup>a</sup>	-29
6.25	6.4 $\pm$ 0.039	6.16 $\pm$ 0.04 <sup>a</sup>	-77 $\pm$ 4	-22 $\pm$ 3 <sup>a</sup>	-5

<sup>a</sup> Difference is significant compared with the corresponding values obtained in the absence of FCCP ( $p < 0.02$ ).

FCCP and the surface-to-volume ratio of the cells. In nerve terminals in which this ratio is high,  $1 \mu M$  FCCP is able to elicit a 30–40-mM increase in  $[Na^+]_i$  during an incubation of a few minutes. In cell cultures or freshly isolated cells with a relatively larger volume, this effect may be slower and require higher FCCP concentrations.

The results presented here emphasize the importance of membrane depolarization resulting from a direct action of protonophores on the plasma membrane and of the elevated  $[Na^+]_i$ , which is secondary to an effect on the mitochondria; these may strongly influence cellular responses to FCCP, particularly when sequestration of  $Ca^{2+}$  loads is involved.

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