Simultaneous Monitoring of Ionophore- and Inhibitor-mediated Plasma and Mitochondrial Membrane Potential Changes in Cultured Neurons

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Although natural and synthetic ionophores are widely exploited in cell studies, for example, to influence cytoplasmic free calcium concentrations and to depolarize in situ mitochondria, their inherent lack of membrane selectivity means that they affect the ion permeability of both plasma and mitochondrial membranes. A similar ambiguity affects the interpretation of signals from fluorescent membrane-permeant cations (usually termed “mitochondrial membrane potential indicators”), because the accumulation of these probes is influenced by both plasma and mitochondrial membrane potentials. To resolve some of these problems a technique is developed to allow simultaneous monitoring of plasma and mitochondrial membrane potentials at single-cell resolution using a cationic and anionic fluorescent probe. A computer program is described that transforms the fluorescence changes into dynamic estimates of changes in plasma and mitochondrial potentials. Exploiting this technique, primary cultures of rat cerebellar granule neurons display a concentration-dependent response to ionomycin: low concentrations mimic nigericin by hyperpolarizing the mitochondria while slowly depolarizing the plasma membrane and maintaining a stable elevated cytoplasmic calcium. Higher ionomycin concentrations induce a stochastic failure of calcium homeostasis that precedes both mitochondrial depolarization and an enhanced rate of plasma membrane depolarization. In addition, the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone only selectively depolarizes mitochondria at submicromolar concentrations. ATP synthase reversal following respiratory chain inhibition depolarizes the mitochondria by 26 mV.

Whereas synthetic and natural ionophores are powerful tools for manipulating cellular physiology, their inherent lack of specificity means that they influence the ion permeability of several membranes in the cell. The two classes of ionophores that are most commonly used are the electroneutral Ca²⁺/H⁺ exchangers typified by ionomycin, and protonophores, such as carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Ionomycin is most commonly used with the assumption that it will generate a stable, moderately elevated, cytoplasmic free Ca²⁺ concentration, [Ca²⁺], with maintained cell viability. However, ionomycin also intercalates into the inner mitochondrial membrane where it provides an additional pathway for Ca²⁺ efflux from the matrix in parallel with the native Ca²⁺/Na⁺ antipporter, setting up a proton-dissipating, i.e. uncoupling, Ca²⁺ cycle that is controlled by the activity of the mitochondrial Ca²⁺ uniporter and hence by [Ca²⁺]. The bioenergetic consequences of this are usually ignored, although they could have profound effects on cellular function. Conversely, protonophores such as FCCP that are widely employed to depolarize mitochondria in intact cells can affect plasma membrane potentials at higher concentrations.

An equally important ambiguity surrounds the widespread use of cationic, membrane permeant, fluorescent probes as mitochondrial membrane potential indicators (3–5) because their uptake and equilibrium accumulation within the mitochondrial matrix of the cell is responsive equally to the plasma membrane potential, Δψₚ, and the mitochondrial membrane potential, Δψₚₘ. With the explosion of interest in the multiple roles played by in situ mitochondria in cell physiology and pathology (reviewed in Refs. 6 and 7) has come the importance of accurately monitoring changes in Δψₚₘ in intact cells. The complicating role of the plasma membrane was recognized at an early stage (4) and is exacerbated in studies in which both Δψₚ and the plasma membrane potential (Δψₚ) change during the experiment. We have previously presented a semiquantitative technique for the interpretation of whole cell cationic indicator traces under such conditions (8) based on curve fitting and estimates of likely changes in Δψₚ. The approach has proven useful for deciphering experiments in “quench mode” (when the probe concentration in the matrix is sufficient for reversible aggregation) as well as for interpreting traces obtained with probes with differing permeability rate constants (8–11). Quench mode is only applicable to experiments in which rapid step changes in Δψₚ occur while the cell is being imaged (8). Conversely, low probe loadings that avoid matrix quenching must be employed to follow slow changes in potential as well as to estimate pre-existing values of Δψₚₘ in cell populations (reviewed in Ref. 12). Under these latter conditions there is serious ambiguity as to whether the observed change in fluorescence is due to a difference in Δψₚ, Δψₚₘ, or both.

It is evidently important to monitor both potentials. Anionic membrane-permeant probes are excluded from polarized cells due to the negative plasma membrane potential but partition increasingly into the cell upon plasma membrane depolarization. Because of their negative charge these probes are not accumulated by mitochondria. Anionic oxonol dyes have been used for several years to monitor changes in Δψₚ (13–16), but their usefulness is compromised by a relatively slow equilibration across the plasma membrane. Recently a proprietary plasma membrane potential assay kit (Molecular Devices, Sunnyvale, CA) has become available in which the problem of background fluorescence
from the high extracellular probe concentration is suppressed by a hydrophilic quencher (17).

In this paper a technique was developed for combining the use of the Molecular Devices fluorescent anion (which is termed PMPI, for “plasma membrane potential indicator”) with the established cationic indicator tetramethylrhodamine methyl ester (TMRM”). At the same time we have devised a curve-fitting spreadsheet to interpret the traces. As well as providing a more quantitative means of compensating the TMRM” signal for changes in Δψm, this combined technique allows for the first time simultaneous and continuous monitoring of Δψm and Δψn in cultured neurons. Whereas the technique is validated for cerebellar granule neurons exposed to a variety of ionophores and inhibitors on a confocal microscope, with suitable calibration the methodology is equally applicable to any attached cell preparation and can be used with non-confocal imaging. Finally the curve-fitting spreadsheet may also be used for single-probe studies of Δψn, where Δψp is invariant or its changes can be estimated.

MATERIALS AND METHODS

Reagents—TMRM”, fluo-4 and fluo-5F were from Molecular Probes (Eugene, OR). PMPI is a proprietary component of the Membrane Potential Assay Kit (R-8042) from Molecular Devices Corp., Sunnyvale, CA. All other reagents were from Sigma.

Preparation of Cerebellar Granule Neurons—Cerebellar granule neurons were prepared from 7-day-old Wistar rats as previously described (18) with modifications. Briefly, cells were plated into coverslip-based 8-well chambers (LabTek, Naperville, IL) previously coated with 33 g/ml streptomycin and 50 μg/ml penicillin, and 50 μg/ml streptomycin. 24 h after plating, 10 μM cytosine arabinoside was added to inhibit growth of non-neuronal cells. Cultures were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO2, 95% air and used for experiments at 12–14 days in culture.

Plasma Membrane Potential Indicator—An individual vial from a Molecular Devices ”membrane potential assay kit, explorer format” (R-8042) containing a proprietary plasma membrane potential indicator was reconstituted in 1 ml of distilled water, dispersed into 50-μl aliquots, and frozen (PMPI stock). For spectral analysis 20 μl of PMPI stock was diluted with 250 μl of water and extracted with 250 μl of octanoyl alcohol to separate the anionic indicator from the aqueous quencher present in the PMPI stock. Excitation and emission spectra (Fig. 1) were determined with a PerkinElmer LS50 scanning spectrophotometer for PMPI in octanoyl alcohol and compared with tetramethylrhodamine in water.

Simultaneous Monitoring of PMPI and TMRM” Fluorescence—Cerebellar granule neurons (CGN) were washed and incubated (37 °C, pH 7.4) for 45 min prior to imaging with a medium (low K-medium) containing 3.5 mM KCl, 120 mM NaCl, 1.3 mM CaCl2, 0.4 mM KH2PO4, 5 mM NaHCO3, 1.2 mM NaSO4, 15 mM D-glucose, 20 mM Na-TES, 1 μM tetraphenylboron, 5 mM TMRM”, and 0.5 μM/ml PMPI stock. An identical medium in which 120 mM NaCl was substituted by 120 mM KCl was prepared (high-K medium). 5 mM TMRM” is below the limit for probe aggregation and quenching within the matrix. In some experiments the TMRM” concentration was varied. The PMPI concentration was chosen to obtain a signal comparable with that of TMRM”. The presence of tetraphenylboron (TPB”) facilitates the equilibration of TMRM” and other lipophilic cations (4, 8, 19) across the plasma membrane. No effect of 1 μM TPB” on the equilibrium distribution of either PMPI or TMRM” was detected. No extracellular PMPI fluorescence could be detected and addition of 0.5% Triton X-100 abolished both the TMRM” and PMPI signals associated with the cells.

Cells were imaged on a Zeiss Pascal confocal Axiovert 100M microscope equipped with a computer-driven stage. The technique is equally applicable to non-confocal imaging, and unless otherwise stated, the pinhole diameter was increased to give an optical slice of 10 μm to allow collection of the defocused signal from individual somata. Fluorescence was excited in single track mode with the 514-nm band of an argon laser. Because the emission peaks of the two indicators are separated by only 20 nm (Fig. 1) the spectral overlap must be corrected. To accomplish this the emitted epifluorescence was split with a 570-nm dichroic mirror and detected in channel 1 through a Chroma 595–650-nm filter and channel 2 with a Chroma 525–575-nm filter. Crossover between the two channels was quantified with cell preparations loaded with a single probe. The values obtained were dependent upon the amplifier gains for the two channels and so these were kept constant throughout. Laser power was adjusted to obtain an optimal image and did not exceed 5% when used with the ×20 air objective. Whereas the Zeiss software allows for pixel by pixel correction of crossover between the two emission spectra using its subtractive function, it is equally valid to correct single-cell time courses in a spreadsheet. Scan averaging was performed for the high resolution studies shown in Fig. 2 using the ×63 oil immersion objective with line mode summing of four repetitive scans at 1% laser power to minimize phototoxicity.

Curve-fitting Computer Simulation—The simulation to convert the TMRM” and PMPI fluorescence traces into a time course of changes in Δψp and Δψm. The methodology is equally applicable to any attached cell preparation and can be used with non-confocal imaging. Finally the curve-fitting spreadsheet may also be used for single-probe studies of Δψn, where Δψp is invariant or its changes can be estimated.
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FIGURE 2. Confocal images of CGNs equilibrated with PMPI and 5 mM TMRM. CGNs were equilibrated with PMPI and 5 mM TMRM in low-(3.9 mM) K medium as described under “Materials and Methods.” Images were corrected for crossover between the fluorophores (Equations 1 and 2). After imaging cells in low-K medium, media was exchanged to increase [K\(^+\)] to 25 mM and the field was imaged again when re-equilibration was complete. The lower panels show the intensity profiles through the cell, scale in micrometers. Note the reciprocal changes in probe intensity, the lack of co-localization between the PMPI and TMRM\(^+\) fluorescence, and the exclusion of PMPI from the nucleus.

data points are represented by symbols (closed squares for PMPI, open squares for TMRM\(^+\)) and the fitted computer simulation by the underlying solid lines. The values for \(\Delta \Psi_p\) and \(\Delta \Psi_m\), that are input into the simulation to produce the curve fits are shown in the adjacent graphs.

RESULTS

Fig. 2 depicts equatorial confocal slices through the somata of 12 DIV CGNs equilibrated with PMPI and 5 mM TMRM\(^+\). In the polarized cells, PMPI fluorescence is faint and associated with the plasma membrane with slight cytoplasmic fluorescence. Depolarization with 25 mM K\(^+\) results in a increase in cell-associated PMPI fluorescence following entry of the probe into the cytoplasm although the probe remains excluded from the nucleus. The decrease in TMRM\(^+\) fluorescence is due to the decreased probe accumulation across the plasma membrane in the partially depolarized cells (see Equation 5).

Computer Simulation—The computer simulation can be accessed in supplementary data. The theoretical basis of the simulation is developed below.

PMPI Calibration—It is first necessary to calibrate the enhancement in the PMPI fluorescence as a function of plasma membrane depolarization. This does not follow an ideal Nernstian relationship, but instead is determined empirically by quantifying the fluorescent enhancement obtained when \(\Delta \Psi_p\) is depolarized by increasing KCl concentrations. The relationship between \(\Delta \Psi_p\) and the K\(^+\) concentration of the medium (Fig. 3A) was calculated from electrophysiological data in Laritzen et al. (20) applying the Goldman-Hodgkin-Katz equation. An initial value for the \(\Delta \Psi_p\) of 8–12 DIV rat CGNs in 3.9 mM KCl media of \(-83\) mV at 37 °C was calculated. A series of experiments were then performed with PMPI-equilibrated CGNs in which step increases in K\(^+\) concentrations were made by removing defined volumes of low-K medium and replacing this with an equal volume of high-K medium to give final K\(^+\) concentrations from 4 to 80 mM. The mean fluorescent enhancement for 10 cell bodies was determined for each K\(^+\) concentration and plotted as a function of \(\Delta \Psi_p\) (Fig. 4A). The best fit with the empirical fluorescent enhancement was obtained with a second-order regression curve,

\[
E = 1 + 0.03(\Delta \Psi_{KCl} - 83) + 0.0005(\Delta \Psi_{KCl} - 83)^2 \quad (\text{Eq. 1})
\]

where \(E\) is the fluorescent enhancement relative to cells in 3.9 mM KCl medium and \(\Delta \Psi_{KCl}\) the calculated plasma membrane potential at a given KCl concentration. It should be noted that the fluorescent enhancement is considerably less than that predicted for the change in free cytoplasmic PMPI concentration from the Nernst equation (dashed line in Fig. 3A), presumably due to complicating factors of probe binding to membranes and proteins and changes in fluorescence yield. Using this empirical relationship, an observed change in whole cell PMPI fluorescence can be calibrated as a function of \(\Delta \Psi_p\).

Single-channel Monitoring of \(\Delta \Psi_p\)—The PMPI probe may be used in the absence of TMRM\(^+\) and tetraphenylboron to monitor changes in \(\Delta \Psi_p\) using the empirical curve fit in Equation 1. In this case the only additional parameter that is required to produce a dynamic read-out of potential is the rate constant for the redistribution of PMPI across the plasma membrane. This is estimated from the kinetics of the PMPI fluorescence enhancement resulting from a step change in \(\Delta \Psi_p\) caused by an increase in KCl concentration. The assumption is made that the rate of change in fluorescence is a first-order function of the disequilibrium between the instantaneous and final probe distribution and is proportional to the rate constant for the PMPI re-equilibration across the plasma membrane,

\[
f(t + \delta t) = f(t) + (f(f) - f(t)) \times \delta t \times k_{PMPI} \quad (\text{Eq. 2})
\]

where \(\delta f/\delta t\) is the rate of increase in signal, \(f(f)\) is the fluorescence at time \(t, f(t)\) is the final fluorescence, and \(k_{PMPI}\) is the rate constant (s\(^{-1}\)). Using this approach, a good curve-fit for the KCl jump is obtained by adopting a rate constant of 0.04 s\(^{-1}\) for PMPI equilibration across the plasma membrane (data not shown). It must be noted that this value refers to the granule cell soma and will differ for other cells.

FIGURE 2. Confocal images of CGNs equilibrated with PMPI and 5 mM TMRM. CGNs were equilibrated with PMPI and 5 mM TMRM in low- (3.9 mM) K medium as described under “Materials and Methods.” Images were corrected for crossover between the fluorophores (Equations 1 and 2). After imaging cells in low-K medium, media was exchanged to increase [K\(^+\)] to 25 mM and the field was imaged again when re-equilibration was complete. The lower panels show the intensity profiles through the cell, scale in micrometers. Note the reciprocal changes in probe intensity, the lack of co-localization between the PMPI and TMRM\(^+\) fluorescence, and the exclusion of PMPI from the nucleus.
resulting from the hyperpolarization produced by the addition of 0.5 mM NMDA. The trend line (solid line) is given by a second-order regression curve (see Equation 6). Neurons were equilibrated with 5 nM TMRM in low-K medium and cell soma were imaged. Myxothiazol (5 μM) plus oligomycin (5 μg/ml) were then added and the final fluorescence determined. PMPI fluorescence confirmed that no change occurred in ΔΨm (see Fig. 7). The vertical lines show the mean (solid line) ± S.E. (dashed lines) for Tdepol/Tpol for 10 randomly chosen cells. The horizontal lines are the corresponding extrapolations to the y axis, indicating a mean matrix fractional volume of 0.023 ± 0.02. Cells were equilibrated in low-K medium for 60 min with the indicated concentrations of TMRM in the absence of TPB+. At the arrow a combination of 2.5 μM rotenone, 2.5 μg/ml oligomycin, and 250 nM FCCP was added to collapse ΔΨm. The transient spike indicates dequenching as aggregated probe is released from the matrix. Each experimental trace is the mean of 10 randomly chosen somata with backgrounds subtracted. C, simulation of experimental traces utilizing a best-fit quench limit of 140 μM.

To validate the responsiveness of PMPI fluorescence to changes in ΔΨm, 12 DIV CGNs were depolarized with NMDA, kainate, or ouabain (data points in Fig. 3, B–D). The computer simulation (detailed in supplementary data) was used to curve-fit the experimental data. The solid lines in Fig. 3, B–D, show the close fit obtained with the experimental data points when the ΔΨm time courses shown in Fig. 3, E–G, are input into the simulation. Comparison with the final KCl depolarization, calculated to decrease ΔΨm to −40 mV, demonstrates how extensive the plasma membrane depolarization is in the cell population and also that in both cases there is a partial recovery of membrane potential, perhaps due to receptor desensitization, during the exposure. It is notable that the repolarization of the plasma membrane following addition of NBQX to terminate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionionic acid/kainate receptor activation is slower than that following NMAD receptor inhibition by MK801. The extent of depolarization occurring during α-amino-3-hydroxy-5-methyl-4-isoxazolepropionionic acid/kainate receptor activation correlates closely with electrophysiological data reported by Kiedrowski and Mienville (21).
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**Single-channel Monitoring of Δψₚ**—The simulation described in the supplementary data is valid for the interpretation of experiments in which just the cationic indicator is present, as long as the plasma membrane potential is constant during the experiment, or changes in Δψₚ can be estimated. The validity extends to traces obtained under non-quench and quench conditions, and with cationic probes that are rapidly permeant (e.g. TMRM⁻ in the presence of tetraphenylboron) or slowly permeant (e.g. rhodamine 123).

**Mathematic Basis of the Simulation**—The basic simplifying assumptions are similar to those previously reported (8).

(i) The equilibrium distribution of TMRM⁻ (and under non-quench conditions its fluorescence) is a simple consequence of the Nernstian distribution of the probe across both the plasma and mitochondrial membranes; thus no corrections are made for binding, non-ideal behavior or spectral changes resulting from probe redistribution (see Ref. 22).

(ii) The matrix volume is assumed to remain constant during an experiment. This is an approximation, because in practice in some conditions such as extensive Ca²⁺ loading, alteration in mitochondrial morphology consistent with matrix swelling can be observed (23).

(iii) As a consequence of the enormous surface/volume ratio of the mitochondrial inner membrane/matrix compared with the plasma membrane/cytoplasm, probes equilibrate much more rapidly across the mitochondrial membrane than across the plasma membrane (8). For these studies it is valid to assume that re-equilibration of TMRM⁻ between matrix and cytoplasm occurs within the sampling interval of the experiment.

Interpretation of the Δψₚ profile underlying changes in the whole cell TMRM⁻ fluorescence is somewhat complex and depends on the initial values of Δψₚ and Δψₘ, the fraction of the cell volume occupied by the mitochondrial matrices, the quench limit (the concentration at which the probe forms non-fluorescent aggregates within the mitochondrial matrix) and, for dynamic determinations, the rate constant for the equilibration of the probe across the plasma membrane. Each of these parameters will now be derived for the cerebellar granule neuron preparations employed in the current study.

**Initial Values of Δψₚ and Δψₘ**—The derivation of an initial value of −83 mV for Δψₚ for CGNs in 3.9 mM K medium at 37 °C has been discussed above. The consensus value for the initial Δψₘ of in situ mitochondria respiring between States 3 and 4 is close to 150 mV, based on semiquantitative data obtained for the mitochondria within isolated nerve terminals (24) and isolated hepatocytes (25) and is consistent with data obtained with isolated mitochondria (see for example, Ref. 26). This initial value is adopted for the simulation. The equilibrium concentration of TMRM⁻ in the cytoplasm (c) and mitochondrial matrix (m) relative to the external medium (e) is given at 37 °C by the following equations.

\[
[TMRM⁺]_m = [TMRM⁺]_c \times 10^{Δψ_m/61} \quad (Eq. 3)
\]

\[
[TMRM⁺]_c = [TMRM⁺]_e \times 10^{Δψ_p/61} \quad (Eq. 4)
\]

\[
[TMRM⁺]_m = [TMRM⁺]_e \times 10^{Δψ_c - Δψ_p/61} \quad (Eq. 5)
\]

The Fraction of the Cell Volume Occupied by the Mitochondrial Matrices—The relative contributions of the cytoplasmic and matrix pools of a cationic probe to whole cell fluorescence will depend on the relative volumes of the two compartments. One way to estimate the volume fraction x of the soma occupied by the mitochondrial matrix is to determine the residual cytoplasmic fluorescence after mitochondrial depolarization by calculating the ratio of the whole cell TMRM⁻ fluorescence (in non-quench mode) for a cell with depolarized mitochondria (e.g. in the presence of myxothiazol to inhibit the respiratory chain and oligomycin to block the ATP synthase) relative to the same cell with polarized mitochondria prior to the addition of inhibitors (Fig. 4A). If the fraction of the cell occupied by the mitochondrial matrices is x then the ratio of the whole cell fluorescence of depolarized versus polarized mitochondria, i.e. \(\frac{\sum TMRM⁻} {\sum TMRM⁺}_{depol} / \frac{\sum TMRM⁺} {\sum TMRM⁺}_{pol}\), will be given as Equation 6.

\[
\frac{\sum TMRM⁻} {\sum TMRM⁺}_{depol} / \frac{\sum TMRM⁺} {\sum TMRM⁺}_{pol} = 1/(1 + x \times 10^{Δψ_m/61}) \quad (Eq. 6)
\]

The ratio determined experimentally in 10 random cells was 0.121 ± 0.01, and substituting this value into Equation 6 gives a value for the volume fraction x of 2.3 ± 0.02% when Δψₚ is 150 mV (Fig. 4A). It must be emphasized that this assumes that there is no potential-independent binding of TMRM⁻ to components of the cell.

For these studies it is valid to assume that re-equilibration of TMRM⁻ between matrix and cytoplasm in response to a step change in Δψₚ occurs within the sampling interval of the experiment. When calculating fluxes across the plasma membrane the cytoplasm plus matrix can thus be considered to be a single compartment. The apparent “volume” of this combined compartment is equivalent to a cytoplasm whose volume is increased by the factor \(1 + x \times 10^{Δψ_m/61}\) from Equation 6. This “expansion factor” is critical for the understanding of the dynamic TMRM⁻ fluorescence response to a change in Δψₚ or Δψₘ. Qualitatively, a step decrease in Δψₘ will decrease the apparent volume and thus increase the concentration in the cytoplasm, leading to redistribution across the plasma membrane to restore the Nernst equilibrium.

**Establishment of the Quench Limit for TMRM⁺ in the Mitochondrial Matrix**—For the simulation to be valid under both non-quench and quench conditions, it is important to establish the concentration of TMRM⁺ in the matrix that initiates aggregation. Cells were equilibrated with TMRM⁺ concentrations from 50 to 10 nM (Fig. 4B). A mixture of rotenone, oligomycin, and FCCP was added to cause a rapid mitochondrial depolarization that is accompanied by a transient “spike” in whole cell fluorescence if sufficient probe was present in the matrix to cause aggregation and quenching. Optimal simulation of these traces using the values determined above for the cell parameters was obtained with a value of 140 µM for the quench limit for TMRM⁺ in the mitochondrial matrix (Fig. 4C).

**Estimation of the Rate Constant for TMRM⁺ Re-equilibration Across the Plasma Membrane**—A minimum estimate for the rate constant in the presence of 1 µM TBP⁻ was obtained by assuming that the collapse of Δψₘ on addition of a 10-fold excess of FCCP (Fig. 8B, cf. A) is fast compared with the sampling interval. By curve fitting the experimental points in Fig. 8B with this proviso, a good fit is obtained with a rate constant not less than 0.015 s⁻¹. It must be emphasized that these values pertain to the somata of rat cerebellar granule cells. Other cells will differ depending on their size, and the probe will redistribute across thin processes more rapidly than into the cell body (8).

**Simultaneous Monitoring of Changes in Δψₚ and Δψₘ by Dual Labeling with PMPI and TMRM⁻**—With appropriate choices of excitation and emission wavelengths it is possible to combine the determinations of Δψₚ and Δψₘ in a single experiment with both probes (see “Materials and Methods”). In the present study this was achieved by exciting at 514 nm and collecting dual emissions at 525–570 and 595–650 nm. Single-track excitation has the advantage that crossover between the channels can be corrected pixel by pixel by the ZEiss software, but crossover could be reduced by exciting PMPI at about 530 nm and TMRM⁺ at 570 nm.
mediated mitochondrial depolarization seen in CGNs upon NMDA receptor activation and the subsequent survival of the cells (8) and in several studies investigating the final catastrophic collapse of $\Delta \psi_m$ in this context (27, 28). However, partial depolarization often produces no change in steady-state signal (Fig. 5C) and studies that only determine initial and final fluorescence can form erroneous conclusions. Quench mode is also relatively insensitive to slow mitochondrial depolarization (Fig. 5G), whereas the response to slow mitochondrial hyperpolarization (not shown) is indistinguishable from that resulting from plasma membrane depolarization (Fig. 5, D and H).

Non-quench mode, typically requiring less than 10 nM TMRM$^+$, is equally applicable to rapid (Fig. 5A) and slow (Fig. 5E) changes in $\Delta \psi_m$, but virtually identical traces are produced by equivalent changes in $\Delta \psi_p$ (Fig. 5, B and F). The parallel monitoring of $\Delta \psi_p$ removes this ambiguity (Fig. 5, A and B and E and F). It should be noted that the PMPI signal faithfully reflects the changes in $\Delta \psi_p$ under all conditions.

Uniprot and Antiport $K^+$ Ionophores Have Opposing Effects on $\Delta \psi_p$ and $\Delta \psi_m$—The $K^+$-uniprot ionophore valinomycin and the $K^+/H^+$ exchange ionophore nigericin are valuable tools in isolated mitochondrial studies to equilibrate, respectively, $\Delta \psi_m$ (29) or $\Delta \psi_p$ (26) with the transmembrane $K^+$ gradient. As with all ionophores, however, their lack of membrane selectivity means that their action in intact cells is complex, particularly because $K^+$ conductances at the plasma membrane play the major role in the maintenance of $\Delta \psi_m$. Valinomycin should slightly hyperpolarize the plasma membrane by bringing $\Delta \psi_p$ closer to the $K^+$ diffusion potential while collapsing the membrane potential of the mitochondria in the high $K^+$ cytoplasm. Fig. 6A shows that both of these changes in potential can be detected by the present methodology. It must be emphasized that valinomycin causes matrix swelling (30), which was not allowed for in the present simulation.

The $K^+/H^+$ ionophore nigericin produces a complex TMRM$^+$ trace (Fig. 6B) that would be difficult to interpret without the parallel monitoring of $\Delta \psi_p$. Curve fitting with the simulation indicates a rapid partial depolarization of the plasma membrane, consistent with the efflux of $K^+$ from the cytoplasm in exchange for protons. This is followed by a mitochondrial hyperpolarization of about 30 mV as the ionophore intercalates into the inner mitochondrial membrane, collapsing $\Delta \psi_p$ and allowing a compensatory increase in $\Delta \psi_m$. This result suggests that the initial mitochondrial $\Delta \psi_p$ is at least ~0.5 pH units (equivalent to 30 mV of proton mototive force).

**Glycolysis Maintains $\Delta \psi_p$ but Lowers $\Delta \psi_m$**—In contrast to experiments performed with isolated mitochondria, respiratory chain inhibition in intact cells does not lead to a total collapse of $\Delta \psi_m$, because the ATP synthase operating in reverse can function as an alternative proton pump driven by the hydrolysis of glycolytically generated ATP and maintain a suboptimal $\Delta \psi_m$. Because the direction and rate of the ATP synthase will be in part governed by thermodynamic disequilibrium, it follows that the $\Delta \psi_m$ maintained by glycolysis will be lower than the $\Delta \psi_p$ generated by respiring mitochondria. Fig. 7A quantifies this difference for the present preparation. The experimental and simulated traces can be superimposed assuming a 26 mV drop in $\Delta \psi_m$ on addition of myxothiazol to inhibit Complex III. Note that $\Delta \psi_p$ is maintained, indicating that glycolysis is sufficiently active to supply ATP for the plasma membrane Na$^+/K^+$-ATPase, the major utilizer of ATP in the neuron, indeed a slight $\Delta \psi_p$ hyperpolarization can be detected. In this and most subsequent figures the average responses of 10 cells are shown. The histogram in Fig. 7A shows the typical cell to cell variability in the membrane potential changes. In contrast, when ATP synthase reversal is prevented by oligomycin (Fig. 7B), myxothiazol initiates a progressive collapse of $\Delta \psi_m$. 

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**FIGURE 6.** The K⁺-uniport ionophore valinomycin and the K⁺/H⁺ antiport ionophore nigericin have opposing effects on CGN $\Delta \psi_m$ and $\Delta \varphi_m$. CGNs were equilibrated in low-K medium with PMPI and TMRM⁺. A, where indicated by the arrow, 0.5 μM valinomycin plus 5 μg/ml oligomycin was added. Note the transient plasma membrane hyperpolarization and the collapse of $\Delta \psi_m$ as the ionophore clamps the mitochondrial membrane potential to the now negligible K⁺ gradient across the inner mitochondrial membrane. B, addition of 0.5 μM nigericin to the cells causes a partial depolarization of the plasma membrane followed by a mitochondrial hyperpolarization of 30 mV, consistent with a collapse of a pH gradient of at least 0.5 units across the inner membrane and the compensatory increase in $\Delta \varphi_m$. Unless otherwise stated, this and subsequent experimental traces are the mean of 10 randomly chosen somata with backgrounds subtracted. In this and subsequent figures, the experimental data points are represented as square data points and the fitted computer simulations by the continuous lines. $\Delta \psi_m$ (sim) and $\Delta \varphi_m$ (sim), membrane potential time courses that generate the fitted computer simulations in A and B.

**Low Micromolar Concentrations of FCCP Depolarize the Plasma Membrane—Protonophores such as FCCP are universally employed to depolarize mitochondria in intact cells. It is established that very high concentrations of protonophores also depolarize the plasma membrane (2), however, it is apparent from Fig. 8 that although 0.25 μM FCCP is selective for the mitochondrial membrane potential, as little as 2.5 μM FCCP initiates a biphasic plasma membrane depolarization that almost totally collapses $\Delta \psi_m$ over a period of 8 min. Whereas the mechanism of this plasma membrane depolarization was not further investigated, it is not due to a failure of mitochondrial ATP production because oligomycin was present in both experiments. It is thus apparent that great care must be taken when titrating in protonophore to intact cell preparations to avoid the metabolic and ionic complications inherent in an unrecognized plasma membrane depolarization.

**Concentration-dependent Effects of Ionomycin on [Ca²⁺]c, $\Delta \psi_m$, and $\Delta \varphi_m$.** The Ca²⁺/2H⁺ exchange ionophores ionomycin, A23187, and 4-bromo-A23187 have been used extensively to raise [Ca²⁺]c, in a great variety of studies. However, with relatively few exceptions (e.g., Refs. 1 and 31) the possible effects on in situ mitochondrial bioenergetics have tended to be ignored. The sequence of events is difficult to predict: an increase in [Ca²⁺]c, as a result of ionophore action at the plasma membrane will increase the activity of the mitochondrial Ca²⁺ uniporter leading to increased uptake into the matrix. However, at the same time the ionophore action at the inner membrane will introduce an additional pathway for Ca²⁺ efflux from the matrix, in parallel with the endogenous Ca²⁺/2Na⁺ exchanger. This enhanced Ca²⁺ cycling is driven by proton re-entry into the matrix and thus begins to “uncouple” the proton circuit in the same way as a conventional protonophore. Indeed in an earlier study we investigated the relationship between [Ca²⁺]c and the respiration of isolated nerve terminals in the presence of increasing ionophore (1). On the other hand, although this Ca²⁺ cycling would be predicted to lower $\Delta \psi_m$, the ionophore additionally collapses the mitochondrial pH gradient (32) that could have a nigericin-like effect (Fig. 6B) of allowing a compensatory increase in $\Delta \varphi_m$. Finally matrix acidification will destabilize any matrix Ca₃PO₄ complex (40).

The dynamic range of individual Ca²⁺ indicators is limited, in the present context this makes it difficult to distinguish between a modest controlled rise in [Ca²⁺]c and an uncontrolled catastrophic Ca²⁺ deregulation as Ca²⁺ entry across the plasma membrane overwhelms the Ca²⁺ influx and sequestration mechanisms of the cell. In Fig. 9 parallel experiments are shown where low ionomycin concentrations are added to CGNs loaded with either the high affinity fluo-4 ($K_d = 345$ nM) or the low affinity fluo-5F ($K_d = 2300$ nM). Ca²⁺ homeostasis was only maintained for most cells during the period of the experiment with the lowest concentration of ionomycin (0.5 μM). With 1 μM ionomycin and fluo-5F (Fig. 9E) a sudden secondary stochastic rise in [Ca²⁺]c is seen and with 3 μM ionophore this Ca²⁺ deregulation is seen in almost all cells. Thus in the entire fields 9% of cells deregulated with 0.5 μM ionomycin, 33% with 1 μM, and 92% with 3 μM ionomycin within 13 min of ionophore addition.

When cells were loaded with both fluo-5F and TMRM⁺ (Fig. 10A) cells imaged during stochastic Ca²⁺ deregulation showed a low [Ca²⁺]c and retained mitochondrial TMRM⁺ labeling (e.g. cell 1), a high [Ca²⁺]c and no TMRM⁺ labeling (e.g. cell 3), or a high fluo-5F fluorescence with a retained TMRM⁺ signal (e.g. cell 2). When the time courses of individual cells was followed it was found that the initiation of the secondary rise in [Ca²⁺]c (a in Fig. 10, B and C) always preceded the initiation of fall in TMRM⁺ fluorescence (b in Fig. 10, B and C). Fig. 10B shows the time interval between these two events for individual cells within a given field.

As discussed earlier, the decrease in TMRM⁺ fluorescence cannot be interpreted without parallel measurement of $\Delta \psi_m$ because it could be a consequence of depolarization of the mitochondrion, the plasma membrane, or both. It is additionally important to relate changes in plasma
and mitochondrial potentials to the biphase increase in [Ca\textsuperscript{2+}]; however, because the spectra of PMPI and fluo-5F superimpose, it is necessary to perform two parallel experiments, one with PMPI and TMRM\textsuperscript{+} and a second with fluo-5F and TMRM\textsuperscript{+}. This analysis demonstrates the two distinct modes of ionomycin action: either a modest elevation in [Ca\textsuperscript{2+}]\textsubscript{c} with maintained mitochondrial integrity, or a stochastic failure of Ca\textsuperscript{2+} homeostasis and resulting mitochondrial depolarization. It is clearly essential to establish which mode is operative when interpreting experiments in which the Ca\textsuperscript{2+} ionophores are added with no independent monitor of mitochondrial integrity.

**DISCUSSION**

**Parallel Monitoring of Plasma and Mitochondrial Membrane Potentials**—All techniques for monitoring changes in **in situ** mitochondrial membrane potentials rely on the interpretation of experiments in which membrane-permeant cations are initially allowed to accumulate across the plasma and mitochondrial membranes. The two modes that are employed for fluorescent cationic indicators, quench mode and non-quench mode, have to be carefully distinguished, because they can give opposing responses to changes in \(\Delta \phi_m\). In quench mode (typically achieved with TMRM\textsuperscript{+} by equilibrating neurons with 50–100 m\textsuperscript{M} probe) aggregation and quenching occurs in the matrix with the result that mitochondrial depolarization results in an increase in whole cell fluorescence as the probe is released into the cytoplasm. Such an increase is transient, however, because the “excess” cytoplasmic probe redistributes to restore the Nernst equilibrium across the plasma membrane. For this reason “quench mode” is best studied with
probes such as rhodamine 123 that are relatively slowly permeant across the plasma membrane (re-equilibration between matrix and cytoplasm is always very rapid due to the enormous surface to volume ratio of the matrix). Rhodamine 123 is, however, difficult to quantify because its low membrane permeability means that cells are usually loaded by brief exposure to very high (i.e. micromolar) concentrations of probe (33). One advantage of quench mode is that cell and mitochondrial depolarizations produce opposite responses that can frequently be distinguished kinetically (8). However, quench mode cannot accurately monitor slow changes in $\Delta \psi_m$ when release from the matrix occurs at a comparable rate to re-equilibration across the plasma membrane (Fig. 5).

Non-quench mode (achieved, for example, with neurons equilibrated with 1–10 nM TMRM$^{\pm}$) can follow slow changes in $\Delta \psi_m$ and distinguish between populations of cells with differing steady-state potentials, but cannot distinguish at whole cell resolution between a change in $\Delta \psi_m$ and $\Delta \psi_p$ (Fig. 5). The present study reveals that significant changes in $\Delta \psi_p$ occur with commonly used ionophores at low micromolar concentrations, such as FCCP (Fig. 8) and ionomycin (Fig. 10), whereas the fluorescence response of cationic indicators to large plasma membrane depolarizations (Figs. 2 and 3) can readily be misinterpreted as reflecting changes in $\Delta \psi_m$ (34).

Anionic oxonol dyes such as di-BAC$_4$ (3) have been used extensively to monitor changes in $\Delta \psi_p$ in cultured cells (e.g. Refs. 35 and 36) although until recently their utility has been limited by sensitivity and rate of re-equilibration. The availability of the proprietary FLIPR (Plasma) Membrane Potential Indicator Kit (37) incorporating an extra-cellular quencher has largely overcome these problems, with its large dynamic range and rapid response. The concentration of probe can be drastically reduced below that recommended to balance the emission intensity with the 5 nM TMRM$^{\pm}$ used in this study with no loss of response. Calibration of the response with varied potassium media and application of the Goldman-Hodgkin-Katz equation is straightforward and the resulting traces can be used both to obtain information on $\Delta \psi_p$ per se and also to correct the TMRM$^{\pm}$ response for changes in plasma membrane potential.

**Uses and Limitations of the Simulation**—In 2000 we (8) published a detailed study in which the mechanistic basis underlying the whole cell fluorescence of probes such as TMRM$^{\pm}$ was investigated. It was possible to devise a simple Excel program based on three principles, probe distribution toward a Nernst equilibrium across both plasma and mitochondrial membranes, much faster equilibration across the inner mitochondrial membrane than across the plasma membrane, and probe aggregation at a critical matrix concentration. Whereas the original simulation has proven valuable for predicting and interpreting the whole cell fluorescence response of cells equilibrated with cationic probes in both quench and non-quench mode (8) the lack of information about changes in $\Delta \psi_p$ is a significant limitation. The present simu-
A number of studies have monitored changes in mitochondrial volume fraction, and rate constants for the two probes across the plasma membrane are determined.

A number of studies have monitored changes in Δψ in intact cells by following the ratio of fluorescent intensity between mitochondria-rich and mitochondria-poor regions of single cells (38, 39). This approach must be used with low non-quenching concentrations of probe, and although in theory this removes the dependence on the plasma membrane potential, there are considerable errors in estimating the tiny difference in fluorescent intensity between the mitochondria-poor cytoplasm and the background (see Fig. 2).

Whereas the combined use of the two indicators provides considerably more precise information than has previously been available, there are a number of limitations. First, as with all such studies, changes in matrix volume are not allowed for, although in the present study the mitochondrial swelling accompanying, for example, valinomycin addition, does not influence the interpretation because the depolarized mitochondria are depleted of TMRM. Second, high quench-mode concentrations of TMRM (+50 nM) appear to interfere with the PMPI response (data not shown). Third, the large correction to the TMRM signal required with the extensive plasma membrane depolarizations caused by excitatory ionotropic receptor activation (Fig. 3) introduces a significant uncertainty in the calculated Δψ. Quench-mode experiments with slowly equilibrating probes remain the best way to detect step changes in Δψ under these special circumstances (8).
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mitochondrial membrane potential in cultured neurons (31), but the mechanism of the depolarization and its relationship to elevated [Ca\(^{2+}\)]\(_{\text{c}}\) is unclear. In addition to its action at the plasma membrane, intercalation of the ionophore into the inner mitochondrial membrane will acidify the matrix (32), destabilize any matrix Ca\(_3\)PO\(_4\) complex (40) and release Ca\(^{2+}\) into the cytoplasm, induce dissipative Ca\(^{2+}\) cycling that can uncouple the mitochondria (1), and ultimately induce a mitochondrial permeability transition (41, 42). The present study clarifies the causal relationship between the elevated [Ca\(^{2+}\)]\(_{\text{c}}\) and mitochondrial during this stochastic Ca\(^{2+}\) deregulation. Prior to deregulation it is possible to detect a mitochondrial hyperpolarization consistent with the decrease in transmembrane pH induced by the ionophore (32).

Importantly, the rapid increase in [Ca\(^{2+}\)]\(_{\text{c}}\) occurs before the plasma membrane has depolarized sufficiently to trigger the activation of voltage-activated Ca\(^{2+}\) channels (Fig. 10, C and D) and before the mitochondria start to depolarize (Fig. 10). Thus mitochondrial bioenergetic failure is a consequence, rather than a cause, of the cytoplasmic Ca\(^{2+}\) deregulation.

Conclusions—The present study is an attempt to improve the precision with which changes in mitochondrial membrane potential can be monitored in intact neurons. We have recently described a “cell respirometer” (43) that allows the respiratory rates of coverslip-attached cells to be continuously monitored and allows calculation of ATP turnover, proton leak, and reserve respiratory capacity. Used in combination these novel techniques may allow in situ mitochondrial bioenergetics to be quantified with a precision approaching that for isolated mitochondria while avoiding the pitfalls in mitochondrial isolation and incubation in a non-physiological environment.

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REFERENCES
1. Åkerman, K. E. O., and Nicholls, D. G. (1981) J. Biol. Chem. 256, 1053–1069
2. Rottenberg, H., and Wu, S. L. (1998) Biochim. Biophys. Acta 1404, 393–404
3. Ehrenberg, B., Montana, V., Wei, M. D., Wuskel, J. P., and Loew, L. M. (1988) Biophys. J. 53, 785–794
4. Farkas, D. L., Wei, M. D., Feddebroiello, P., Carson, J. H., and Loew, L. M. (1989) Biophys. J. 56, 1053–1069
5. Fink, C., Morgan, F., and Loew, L. M. (1998) Biophys. J. 75, 1648–1658
6. Nicholls, D. G. (2004) Curr. Mol. Med. 4, 149–177
7. Duchen, M. R. (2004) Mol. Aspects Med. 25, 365–401
8. Ward, M. W., Rego, A. C., Frenguelli, B. G., and Nicholls, D. G. (2000) J. Neurosci. 20, 7208–7219
9. Rego, A. C., Ward, M. W., and Nicholls, D. G. (2001) J. Neurosci. 21, 1893–1901
10. Rego, A. C., Vesce, S., and Nicholls, D. G. (2001) Cell Death Differ. 8, 995–1003
11. Poppe, M., Reimertz, C., Düssmann, H., Kroh, A. J., Luetjens, C. M., Böckelmann, D., Nieminen, A. L., Kögel, D., and Prehn, J. H. M. (2001) J. Neurosci. 21, 4551–4563
12. Nicholls, D. G., and Ward, M. W. (2000) Trends Neurosci. 23, 166–174
13. Civitelli, R., Reid, I. R., Halstead, L. R., Avioli, L. V., and Hruska, K. A. (1987) J. Cell. Physiol. 131, 434–441
14. Apell, H. J., and Bresch, B. (1987) Biochim. Biophys. Acta 903, 480–494
15. Kiedrowski, L. (2001) Neuroreport 12, 3579–3582
16. Epps, D. E., Wolfe, M. L., and Groppi, V. (1994) Chem. Phys. Lipids 69, 137–150
17. Baxter, D. F., Kirk, M., Garcia, A. F., Raimondi, A., Holmqvist, M. H., Flint, K. K., Bojanic, D., Distefano, P. S., Curtis, R., and Xie, Y. (2002) J. Biomol. Screen 7, 79–85
18. Courtney, M. J., and Nicholls, D. G. (1992) J. Neurochem. 59, 983–992
19. Scott, I. D., and Nicholls, D. G. (1980) Biochem. J. 186, 21–33
20. Lauritzen, I., Zanzouri, M., Honoré, E., Duprat, F., Ehrenguber, M. U., Lazdunski, M., and Patel, A. J. (2003) J. Biol. Chem. 278, 32068–32076
21. Kiedrowski, L., and Mienville, J. M. (2001) Neuroreport 12, 59–62
22. Scaduto, R. C. Jr., and Grotyohann, L. W. (1999) Biochem. J. 365, 469–477
23. Rintoul, G. L., Filiano, A. J., Brocard, J. B., Kress, G. J., and Reynolds, I. J. (2003) J. Neurosci. 23, 7881–7888
24. Kristian, T., and Siesjo, B. K. (1998) Stroke 29, 705–718
25. Maloff, B. L., Scordilis, S. P., and Tedeschi, H. (1978) J. Cell Biol. 78, 214–226
26. Lambert, A. J., and Brand, M. D. (2004) Biochem. J. 382, 511–517
27. Vergun, O., Keelan, J., Khodorov, B. I., and Duchen, M. R. (1999) J. Physiol. (Lond.) 519, 451–466
28. Vergun, O., Sobolevsky, A. I., Yelshansky, M. V., Keelan, J., Khodorov, B. I., and Duchen, M. R. (2001) J. Physiol. (Lond.) 531, 147–163
29. Nicholls, D. G. (1974) Eur. J. Biochem. 50, 305–315
30. Safitulina, D., Vekslar, V., Zharkovsky, A., and Kaasik, A. (2005) J. Cell. Physiol. 206, 347–353
31. Abramov, A. Y., and Duchen, M. R. (2003) Cell Calcium 33, 101–112
32. Abo, D. F. C., Di Benedetto, G., Maglia, F. P., Filippini, L., and Pozzan, T. (2004) J. Biol. Chem. 279, 11521–11529
33. Keelan, J., Vergun, O., and Duchen, M. R. (1999) J. Physiol. (Lond.) 520, 797–813
34. Schinder, A. F., Olson, E. C., Spitzer, N. C., and Montal, M. (1996) J. Neurosci. 16, 6125–6133
35. Yamada, A., Gaja, N., Ohya, S., Muraki, K., Narita, H., Ohwada, T., and Imazumzi, Y. (2001) Ipn. J. Pharmacol. 86, 342–350
36. Düssmann, H., Rehm, H., Kögel, D., and Prehn, J. H. (2003) J. Cell Sci. 116, 525–536
37. Whiteaker, K. L., Gopalakrishnan, S. M., Groebe, D., Shieh, C. C., Warrior, U., Burns, D. J., Coghlan, M. J., Scott, V. E., and Gopalakrishnan, M. (2001) J. Biol. Chem. 276, 305–315
38. Marks, J. D., Boriboun, C., and Wang, J. (2005) J. Neurosci. 25, 6651–6575
39. Toescu, E. C., and Verkhratsky, A. (2000) Pflugers Arch. 440, 941–947
40. Clayes, S., and Nicholls, D. G. (2003) J. Biol. Chem. 278, 19062–19070
41. Dubinsky, J. M., and Levi, Y. (1998) J. Neurosci. Res. 53, 728–741
42. Petersén, Å., Castilho, R. F., Hansson, O., Wieloch, T., and Brundin, P. (2000) Brain Res. 857, 20–29
43. Jekabsons, M. B., and Nicholls, D. G. (2004) J. Biol. Chem. 279, 32989–33000