

# Cyclosporin A-insensitive Permeability Transition in Brain Mitochondria

INHIBITION BY 2-AMINOETHOXYDIPHENYL BORATE\*

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**The mitochondrial permeability transition pore (PTP) may operate as a physiological  $\text{Ca}^{2+}$  release mechanism and also contribute to mitochondrial deenergization and release of proapoptotic proteins after pathological stress, e.g. ischemia/reperfusion. Brain mitochondria exhibit unique PTP characteristics, including relative resistance to inhibition by cyclosporin A. In this study, we report that 2-aminoethoxydiphenyl borate blocks  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in isolated, non-synaptosomal rat brain mitochondria in the presence of physiological concentrations of ATP and  $\text{Mg}^{2+}$ .  $\text{Ca}^{2+}$  release was not mediated by the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger or by reversal of the uniporter responsible for energy-dependent  $\text{Ca}^{2+}$  uptake. Loss of mitochondrial  $\text{Ca}^{2+}$  was accompanied by release of cytochrome *c* and pyridine nucleotides, indicating an increase in permeability of both the inner and outer mitochondrial membranes. Under these conditions,  $\text{Ca}^{2+}$ -induced opening of the PTP was not blocked by cyclosporin A, antioxidants, or inhibitors of phospholipase  $\text{A}_2$  or nitric-oxide synthase but was abolished by pretreatment with bongkrekic acid. These findings indicate that in the presence of adenine nucleotides and  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ -induced PTP in non-synaptosomal brain mitochondria exhibits a unique pattern of sensitivity to inhibitors and is particularly responsive to 2-aminoethoxydiphenyl borate.**

Mitochondria are temporo-spatial modulators of cytosolic  $[\text{Ca}^{2+}]$  through their ability to both sequester and release  $\text{Ca}^{2+}$  in concert with  $\text{Ca}^{2+}$  transport across the endoplasmic reticulum (1, 2). Physiological mitochondrial  $\text{Ca}^{2+}$  efflux, including that caused by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (mCICR),<sup>1</sup> is attributed to either activation of the permeability transition pore (PTP) (3, 4) or to reversal of the  $\text{Ca}^{2+}$  uniporter (5). Opening of the PTP has been implicated as a mediator of apoptotic and necrotic cell death as well as a regulator of normal cell  $\text{Ca}^{2+}$  homeostasis (4, 6–12). The characteristic traits of the PTP

include reversibility (13), sensitivity to inhibition by cyclosporin A (14), and mitochondrial swelling associated with loss of matrix solutes (15–17). Cyclosporin A is not always effective, however, at inhibiting  $\text{Ca}^{2+}$ -induced mitochondrial swelling and loss of  $\Delta\Psi$  (18–22). In addition, the observation that normal intracellular concentrations of adenine nucleotides and  $\text{Mg}^{2+}$  partially or completely block PTP activation (23, 24) casts doubt on a physiological role of the PTP.

The extent to which mitochondria swell in response to accumulation of large  $\text{Ca}^{2+}$  loads also varies considerably with experimental conditions and with mitochondrial tissue type (25–27). Brain mitochondria are particularly resistant to  $\text{Ca}^{2+}$ -induced swelling (25, 28); moreover, they represent many cell populations, which may explain their heterogeneous response to large  $\text{Ca}^{2+}$  loads and to inhibitors of the PTP, e.g. cyclosporin A (26, 29).

In this study we report that 2-APB (30) prevents  $\text{Ca}^{2+}$ -induced PTP in non-synaptosomal brain mitochondria in the presence of physiological concentrations of ATP and  $\text{Mg}^{2+}$ . 2-APB is reported to inhibit  $\text{Ca}^{2+}$  efflux from mitochondria *in situ* in Jurkat T cells following stimulation of cellular  $\text{Ca}^{2+}$  influx through activation of capacitative  $\text{Ca}^{2+}$  entry (31). Under these conditions, mitochondria transiently accumulate  $\text{Ca}^{2+}$ , followed by release upon restoration of basal cytosolic  $\text{Ca}^{2+}$  levels. The investigators hypothesized that the inhibition of mitochondrial  $\text{Ca}^{2+}$  efflux by 2-APB is because of inhibition of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (32). Our results do not provide evidence for 2-APB inhibition of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger but do indicate that mCICR via the PTP is effectively inhibited. This effect of 2-APB on isolated non-synaptosomal brain mitochondria is observed both in the absence of adenine nucleotides, where inhibition of  $\text{Ca}^{2+}$ -induced swelling is apparent, and in the presence of millimolar concentrations of ATP and magnesium, where swelling is not observed but where  $\text{Ca}^{2+}$  induces release of matrix metabolites and cytochrome *c*. In the presence of ATP, 2-APB-inhibitable  $\text{Ca}^{2+}$ -induced mitochondrial alterations are not affected by most other PTP inhibitors, including cyclosporin A.

## EXPERIMENTAL PROCEDURES

**Isolation of Mitochondria**—Non-synaptic adult rat brain mitochondria were isolated on a Percoll gradient as described previously (33) with minor modifications. Male 300–350-g Sprague-Dawley rats were used for this study. All animal procedures were carried out according to the National Institutes of Health and the University of Maryland, Baltimore, animal care and use committee guidelines. Rats were sacrificed, and forebrains were rapidly removed, chopped, and homogenized in ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM Hepes, and 1 mM EGTA with the pH adjusted to 7.4 using KOH. The brain homogenate was centrifuged at  $1,250 \times g$  for 3 min; the pellet was discarded, and the supernatant was centrifuged at  $20,000 \times g$  for 10 min. The pellet was resuspended in 15% Percoll (Sigma) and layered

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<sup>1</sup> The abbreviations used are: mCICR, mitochondrial  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release; PTP, permeability transition pore; 2-APB, 2-aminoethoxydiphenyl borate; TMRE, tetramethylrhodamine ethyl ester perchlorate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone;  $\Delta\Psi$ , mitochondrial membrane potential; SOCE, store-operated  $\text{Ca}^{2+}$  entry; TRP, transient receptor potential.

on a preformed Percoll gradient (40 and 23%). After centrifugation at  $30,000 \times g$  for 6 min, the mitochondrial fraction located at the interface of the lower two layers was removed, diluted with isolation buffer, and centrifuged at  $16,600 \times g$  for 10 min. The supernatant was discarded, and the loose pellet was resuspended in isolation buffer and centrifuged at  $6700 \times g$  for 10 min. The resulting pellet was suspended in  $100 \mu\text{l}$  of isolation medium devoid of EGTA.

**Mitochondrial  $\text{Ca}^{2+}$  Uptake**—Mitochondrial-dependent removal of medium  $\text{Ca}^{2+}$  was followed using the impermeant pentapotassium salt of the ratiometric dye Fura 6F (Molecular Probes, Portland, OR, USA). Fura 6F (250 nM) was added to a medium containing mitochondria (0.25 mg/ml) and 125 mM KCl, 20 mM Hepes, 2 mM  $\text{KH}_2\text{PO}_4$ , 1  $\mu\text{M}$  EGTA, 4 mM  $\text{MgCl}_2$ , 3 mM ATP, 5 mM malate, and 5 mM glutamate with the pH adjusted to 7.08 with KOH. According to calculations using the Winmaxc software (34), the free  $[\text{Mg}^{2+}]$  under these conditions is  $\sim 1$  mM. It was necessary to use Suprapur KCl (Merck) to minimize  $\text{Ca}^{2+}$  contamination, thereby also minimizing the amount of added EGTA necessary to eliminate deleterious effects of background  $\text{Ca}^{2+}$ . All experiments were performed at  $37^\circ\text{C}$ . Fluorescence intensity was measured in a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan) using 340/380-nm excitation and 510-nm emission wavelengths. The  $[\text{Ca}^{2+}]$  in the medium was calculated using the ratio calibration approach described by Grynkiewicz *et al.* (35). The  $K_d$  for Fura 6F was estimated to be  $2.47 \mu\text{M}$  using the calcium calibration buffer kit number 3 (Molecular Probes). An essentially identical value was obtained using the calcium calibration buffer kit number 2 with magnesium (Molecular Probes).

**Mitochondrial Membrane Potential**—Mitochondrial membrane potential was qualitatively assessed by TMRE (Molecular Probes) fluorescence intensity measured in a Hitachi F-2500 fluorescence spectrophotometer using 549- and 580-nm wavelengths for excitation and emission, respectively ( $37^\circ\text{C}$ ). Because of the small Stokes shift, we assessed the effect of light scatter on the fluorescent signal, and it was found to be less than 10% as compared with TMRE fluorescence. 125 nM dye was added to a medium containing mitochondria (0.25 mg/ml) and 125 mM KCl, 20 mM Hepes, 2 mM  $\text{KH}_2\text{PO}_4$ , 1  $\mu\text{M}$  EGTA, 4 mM  $\text{MgCl}_2$ , 3 mM ATP, 5 mM malate, and 5 mM glutamate with the pH adjusted to 7.08 using KOH.

**Oxygen Consumption**—Mitochondrial respiration was recorded at  $37^\circ\text{C}$  with a Clark-type oxygen electrode (Hansatech, UK). The incubation medium contained 125 mM KCl, 20 mM Hepes, 2 mM  $\text{KH}_2\text{PO}_4$ , 1  $\mu\text{M}$  EGTA, 1 mM  $\text{MgCl}_2$ , 0.8 mM ADP, 5 mM malate, and 5 mM glutamate (or 5 mM succinate plus 1  $\mu\text{M}$  rotenone) with the pH adjusted to 7.08 with KOH. In some experiments as indicated 5 mM succinate (plus 1  $\mu\text{M}$  rotenone) replaced malate plus glutamate as the oxidizable substrate. State 3 (phosphorylating) respiration was initiated by the addition of mitochondria (0.5 mg/ml) to the incubation medium. State 3 respiration was terminated and State 4 (resting) respiration was initiated by the addition of 2  $\mu\text{M}$  oligomycin.

**Cytochrome *c* Release from Mitochondria**—Aliquots of mitochondrial suspensions were taken at specified incubation times, and the mitochondria were separated from the suspending medium by centrifugation at  $14,000 \times g$  for 3 min. The supernatant was carefully removed, and both the supernatant and mitochondrial pellet fractions were immediately frozen and stored at  $-20^\circ\text{C}$ . Cytochrome *c* immunoreactivity was quantified in both fractions using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Before measurement, the supernatant and pellet samples were diluted 1:40 and 1:80, respectively. The release of cytochrome *c* from mitochondria is expressed as the content of cytochrome *c* in the supernatant as a percentage of the total content of cytochrome *c* present in the supernatant plus pellet.

**$\text{NAD}^+$  + NADH Release from Mitochondria**—Aliquots of mitochondrial suspensions were taken at specified incubation times, and mitochondria were separated from the suspending medium by centrifugation at  $14,000 \times g$  for 3 min. The supernatant was carefully removed, and both the supernatant and mitochondrial pellet fractions were immediately frozen and stored at  $-20^\circ\text{C}$ .  $\text{NAD}^+$  plus NADH extraction from both fractions was performed according to the method described by Klingenberg (36). Extracts were transferred to 2 ml of assay medium maintained at  $30^\circ\text{C}$  containing 0.2 mg of alcohol dehydrogenase (Sigma), 50 mM Tris-HCl, 0.6 M ethanol, 50 mM  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$ , pH 7.8. NADH fluorescence was followed in a Hitachi F-2500 fluorescence spectrophotometer using 340- and 460-nm wavelengths for excitation and emission, respectively.

**Mitochondrial Swelling**—Swelling of isolated mitochondria was assessed by measuring light scatter at 660 nm ( $37^\circ\text{C}$ ) in a Hitachi F-2500 fluorescence spectrophotometer. Mitochondria were added at a final concentration of 0.25 mg/ml to 2 ml of medium containing 125 mM KCl, 20 mM Hepes, 2 mM  $\text{KH}_2\text{PO}_4$ , 1  $\mu\text{M}$  EGTA, 1 mM  $\text{MgCl}_2$ , 5 mM malate,

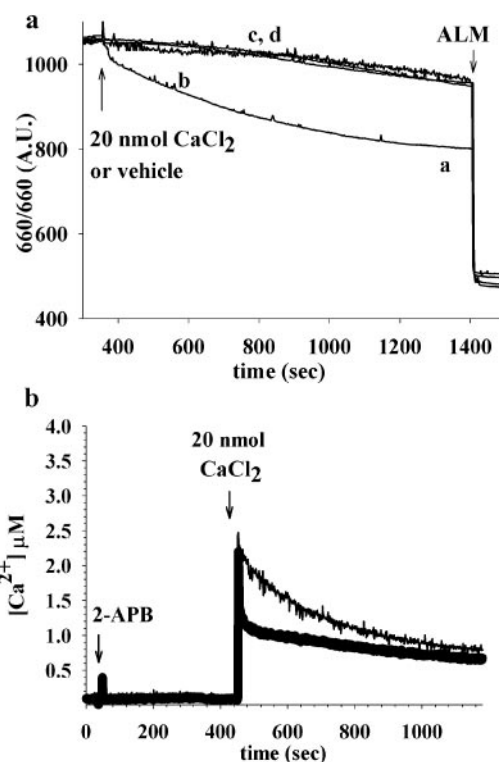


FIG. 1. *a*, effect of 2-APB versus cyclosporin A versus vehicle (ethanol) on the calcium-induced mitochondrial swelling in the absence of adenine nucleotides, estimated by light scatter at 660/600 nm. Shown are 2-APB (100  $\mu\text{M}$ , curve *c*) or vehicle (2  $\mu\text{l}$ , without the subsequent addition of calcium (curve *d*)) with the subsequent addition of calcium (curve *a*) or cyclosporin A (2  $\mu\text{M}$ , curve *b*) added at 50 s and 20 nmol of  $\text{CaCl}_2$  added at 350 s. Alamethicin (ALM), 80  $\mu\text{g}$ , was added at 1400 s. The graph is typical and is one of three independent experiments. *b*, effect of 2-APB on mitochondrial  $\text{Ca}^{2+}$  uptake in the absence of adenine nucleotides. Thick line, 2-APB (100  $\mu\text{M}$ ) added at 50 s and 20 nmol  $\text{CaCl}_2$  added at 450 s. Thin line, 20 nmol of  $\text{CaCl}_2$  added at 450 s. The graph is typical and is one of three independent experiments. A.U., arbitrary units.

and 5 mM glutamate with the pH adjusted to 7.08 with KOH. At the end of each experiment, the non-selective pore-forming peptide alamethicin (80  $\mu\text{g}$ ) was added as a calibration standard to cause maximal swelling.

**Reagents**—Standard laboratory chemicals were from Sigma. 2-APB (Sigma), CGP 37157 (Calbiochem), cyclosporin A (Sigma), bongkreikic acid (Calbiochem), alamethicin (Sigma), tamoxifen (Sigma), spermine (Sigma), xestospongine C (Calbiochem), inositol 1,4,5-triphosphate (ICN), thapsigargin (Calbiochem), trifluoperazine (Sigma), bromoenol lactone (Sigma), aristolochic acid (Biomol), bovine serum albumin (Sigma), 7-nitroindazole (Calbiochem), 1400W (Calbiochem), ubiquinone 0 (Sigma), butylated hydroxytoluene (Sigma), *N*-acetylcysteine (Sigma), catalase (Sigma), and superoxide dismutase (Sigma).

## RESULTS

**2-APB Protects against  $\text{Ca}^{2+}$ -induced Mitochondrial Swelling in the Absence of Adenine Nucleotides**— $\text{Ca}^{2+}$ -induced swelling of brain mitochondria can be demonstrated, but typically, only if adenine nucleotides are omitted from the medium (26). Under these conditions, non-synaptosomal brain mitochondria exposed to 20 nmol of  $\text{Ca}^{2+}$  ( $40 \text{ nmol mg}^{-1}$  protein) (Fig. 1*a*, curve *a*) exhibited a gradual decline in absorbance at 660 nm greater than that observed in the absence of added  $\text{Ca}^{2+}$  (Fig. 1*a*, curve *d*). The presence of cyclosporin A (2  $\mu\text{M}$ ) completely eliminated the  $\text{Ca}^{2+}$ -dependent change in light scattering, indicating involvement of the PTP (Fig. 1*a*, curve *b*). 2-APB (100  $\mu\text{M}$ ) also eliminated the  $\text{Ca}^{2+}$ -dependent light scattering (Fig. 1*a*, curve *c*). Because agents that block mitochondrial  $\text{Ca}^{2+}$  uptake also block mitochondrial swelling without necessarily affecting the PTP, the effect of 2-APB on mitochondrial  $\text{Ca}^{2+}$  uptake and release was measured. After the addition of 20

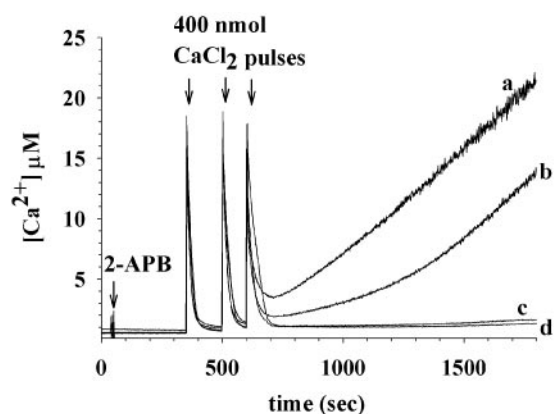


FIG. 2. Dose-dependent effect of 2-APB on mCICR followed by Fura 6F fluorescence (calibrated data). 2-APB was added at 50 s, and 400 nmol of  $\text{CaCl}_2$  added at 350, 500, and 600 s. *a*, no 2-APB added; *b*, 10  $\mu\text{M}$  2-APB; *c*, 50  $\mu\text{M}$  2-APB; *d*, 100  $\mu\text{M}$  2-APB. The graph is typical and is one of four independent experiments.

nmol of  $\text{Ca}^{2+}$  to the mitochondrial suspension,  $\text{Ca}^{2+}$  uptake was faster in the presence of 2-APB than in its absence (Fig. 1*b*, thick versus thin line). Thus, in the absence of adenine nucleotides, both cyclosporin A and 2-APB are effective inhibitors of the  $\text{Ca}^{2+}$ -induced PTP in brain mitochondria.

**Dose-dependent Inhibition of Mitochondrial  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  Release by 2-APB in the Presence of ATP**—After a demonstration that 2-APB is effective at inhibiting PTP in the absence of adenine nucleotides, subsequent experiments were performed in the presence of 3 mM ATP to model more physiologically relevant conditions. In the presence of ATP, brain mitochondria completely accumulated two additions of 400 nmol of  $\text{Ca}^{2+}$  and almost sequestered the third addition. This net uptake of more than 2000 nmol of  $\text{Ca}^{2+}$   $\text{mg}^{-1}$  mitochondrial protein was followed by a slow release of  $\text{Ca}^{2+}$  back to the medium (Fig. 2, curve *a*). Mitochondria suspended in the presence of either 50 or 100  $\mu\text{M}$  2-APB accumulated the entire  $\text{Ca}^{2+}$  load (2400 nmol  $\text{mg}^{-1}$ ) without releasing  $\text{Ca}^{2+}$  during the course of the 20-min experiment (Fig. 2, curves *c* and *d*, respectively). The presence of even 10  $\mu\text{M}$  2-APB resulted in some inhibition of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (Fig. 2, curve *b*).

Fura 6F fluorescence responds reliably to  $[\text{Ca}^{2+}]_i$  in the range of 0.5–50  $\mu\text{M}$  (not shown). Each addition of  $\text{Ca}^{2+}$  to the mitochondrial suspension was a total of 200  $\mu\text{M}$ ; however, according to Winmaxc software (34), the calculated free  $[\text{Ca}^{2+}]_i$  was <100  $\mu\text{M}$  in the presence of  $\text{Mg}^{2+}$  and ATP. Therefore, the peak of the calcium signal before the onset of calcium uptake by mitochondria was underestimated. However, the method used (manual additions of calcium in a cuvette system) is limited in the temporal dimension of the millisecond scale, where much of the calcium uptake takes place, given the enormous avidity of the uniporter ( $V_{\text{max}} > 1200$  nmol  $\text{Ca}^{2+}/\text{mg}$  of protein/min) and/or the rapid mode of uptake (37). It is to be noted that even though the upper range of the bulk  $[\text{Ca}^{2+}]_i$  in cells reported by Fura 2 (or higher  $K_d$  derivatives) fluorescence is in the relatively low micromolar range (38), free  $[\text{Ca}^{2+}]_i$  may well reach the ~100  $\mu\text{M}$  range in certain microdomains (39) such as the perimitochondrial environment (40), (41).

**Comparison of 2-APB to Cyclosporin A and Bongkreikic Acid**—Although cyclosporin A was effective at inhibiting the PTP in brain mitochondria in the absence of adenine nucleotides (Fig. 1*a*), it failed to inhibit mCICR in the presence of ATP (Fig. 3, curve *b*). Bongkreikic acid, another PTP inhibitor, exhibited an ability to inhibit mCICR in the presence of ATP (Fig. 3, curve *c*) that was very similar to that of 2-APB (Fig. 3, curve *d*). Because the vehicle for bongkreikic acid is 1 M  $\text{NH}_4\text{OH}$ , an

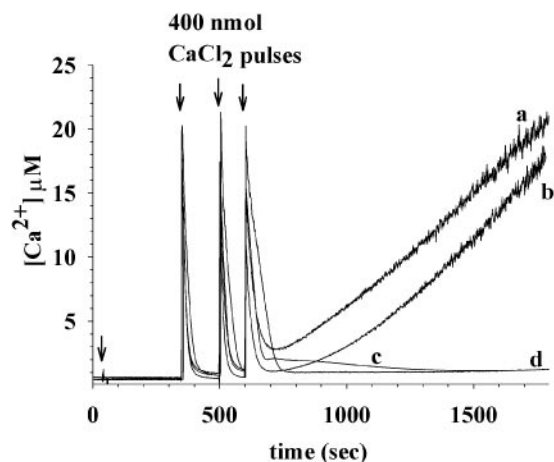


FIG. 3. Effect of 2-APB (100  $\mu\text{M}$ ) and bongkreikic acid (20  $\mu\text{M}$ ) versus cyclosporin A (2  $\mu\text{M}$ ) on mCICR followed by Fura 6F fluorescence (calibrated data). The additions of the drugs are indicated by the arrows. 2-APB, cyclosporin A, or bongkreikic acid were added at 50 s; 400 nmol of  $\text{CaCl}_2$  added at 350, 500, and 600 s. *a*, no additions; *b*, 2  $\mu\text{M}$  cyclosporin A; *c*, 20  $\mu\text{M}$  bongkreikic acid; *d*, 100  $\mu\text{M}$  2-APB. The graph is typical and is one of four independent experiments.

equal volume of vehicle was tested and found to have no effect on  $\text{Ca}^{2+}$  uptake and retention (not shown). Although 2-APB inhibited mCICR, it did cause a slight reduction in the rate of  $\text{Ca}^{2+}$  uptake, particularly after the third addition of  $\text{Ca}^{2+}$  (Fig. 3, curve *d*). The effects of 2-APB on mitochondrial bioenergetics were further analyzed using measurements of both mitochondrial membrane potential and  $\text{O}_2$  consumption.

**Protection by 2-APB against  $\text{Ca}^{2+}$ -induced Loss of Mitochondrial Membrane Potential**—Fig. 4 provides a qualitative evaluation of  $\Delta\Psi$  after the addition of high  $\text{Ca}^{2+}$  loads in the absence and presence of 2-APB (100  $\mu\text{M}$ ) using the fluorescent dye TMRE. In the absence of 2-APB, TMRE fluorescence stabilized within 2 min after the addition of mitochondria to the medium (Fig. 4*a*). The subsequent addition of 400 nmol of  $\text{Ca}^{2+}$  caused an immediate increase in fluorescence, *i.e.* decrease in  $\Delta\Psi$ , as expected because of the collapse of  $\Delta\Psi$  during rapid  $\text{Ca}^{2+}$  influx. After the ~100-s period, during which added  $\text{Ca}^{2+}$  was accumulated, the TMRE fluorescence decreased but remained much higher than the original fluorescence in the absence of  $\text{Ca}^{2+}$ . A similar pattern was observed after the second addition of  $\text{Ca}^{2+}$ . After the third addition, the TMRE fluorescence failed to return toward base line and gradually increased for many minutes thereafter. When 2-APB was added to the mitochondrial suspension (Fig. 4*b*), TMRE fluorescence increased, indicating some reduction in  $\Delta\Psi$  by this compound. However, unlike what was observed in the absence of 2-APB, the subsequent addition of three pulses of  $\text{Ca}^{2+}$  were followed by a return of TMRE fluorescence toward, and eventually to the level maintained before these additions. Thus, at 800 s of incubation,  $\Delta\Psi$  approached complete depolarization in the absence of 2-APB but was at least partially retained in its presence. These observations are consistent with the onset of mCICR in the absence but not in the presence of 2-APB, also observed at ~800 s of incubation. Although 2-APB by itself causes partial mitochondrial depolarization, this effect was not strong enough to impair net  $\Delta\Psi$ -dependent  $\text{Ca}^{2+}$  uptake. It must be emphasized that because of the Nernst equation, data from potentiometric dyes follow a logarithmic, not linear relationship (42). The observation that is intended for demonstration on this experiment is that mitochondria depolarize further upon completion of  $\text{Ca}^{2+}$  uptake (Fig. 4*a*) unless they are pre-treated with 2-APB (Fig. 4*b*). The onset of depolarization upon high  $\text{Ca}^{2+}$  loading (~700 s) coincides exactly with the onset of

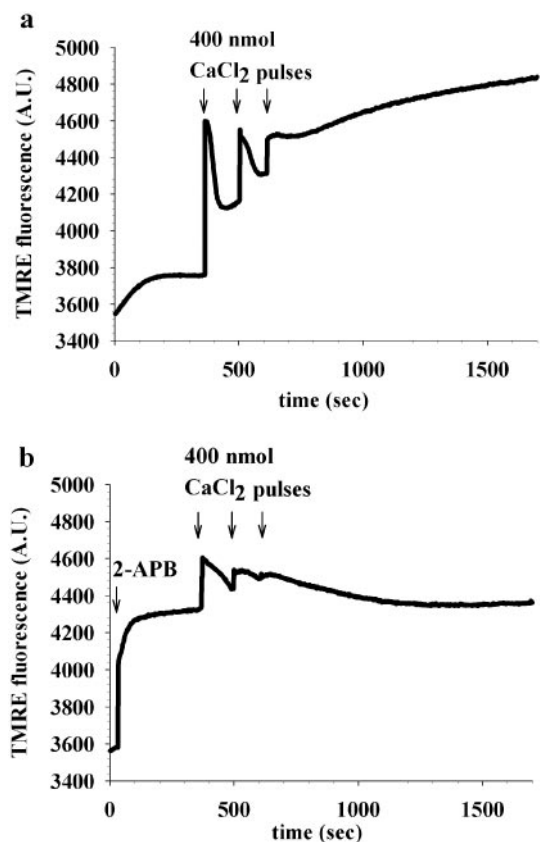


FIG. 4. *a*, effect of high calcium loading on  $\Delta\Psi$  based on TMRE fluorescence. 400 nmol of  $\text{CaCl}_2$  added at 350, 500, and 600 s. The graph is typical and is one of four independent experiments. *b*, effect of pretreatment of 2-APB (100  $\mu\text{M}$ ) and high calcium loading of mitochondria on  $\Delta\Psi$  based on TMRE fluorescence. 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s. The graph is typical and is one of four independent experiments. A.U., arbitrary units.

$\text{Ca}^{2+}$  release (Fig. 2, curve *a*). The choice of the membrane potential-sensitive probe was critical. Many of the available probes were tested (safranin O, tetramethylrhodamine methyl ester perchlorate (TMRM), rhodamine 123, JC-1), but they all exhibited serious drawbacks due to the necessity of high calcium loading; *i.e.* safranin O reduced maximal calcium uptake capacity considerably, as has also been found by other laboratories (43), TMRM and rhodamine 123 were very strong respiratory inhibitors and uncouplers even at low concentrations (20 nM, not shown), affecting respiration more than TMRE although the opposite has been observed for rat heart mitochondria (44), and JC-1 gave an unsatisfactory signal-to-noise ratio but no suppression of respiratory control (not shown).

**Inhibition of  $\text{Ca}^{2+}$ -induced Mitochondrial Cytochrome *c* and Pyridine Nucleotide Release by 2-APB**—Release of the mitochondrial intermembrane protein cytochrome *c* typically accompanies the osmotic swelling and rupture of the outer membrane evoked by activation of the PTP (45–47). Loss of mitochondrial matrix pyridine nucleotides is another measure of the PTP, distinguishing it from a mechanism of depolarization mediated by activation of a “low conductance” pore (48). Fig. 5 provides the results of enzyme-linked immunosorbent assay determinations of cytochrome *c* released into the medium after the addition of  $\text{Ca}^{2+}$  and a comparison of the effectiveness of 2-APB and cyclosporin A in inhibiting this release. In the absence of added  $\text{Ca}^{2+}$ , less than 10% of total mitochondrial cytochrome *c* was lost to the suspending medium after 1800 s of incubation. After the addition of 2400 nmol of  $\text{Ca}^{2+}$   $\text{mg}^{-1}$  protein,  $\sim 58\%$  of total cytochrome *c* was released at 1800 s. No net release was observed at 700 s, immediately before net  $\text{Ca}^{2+}$

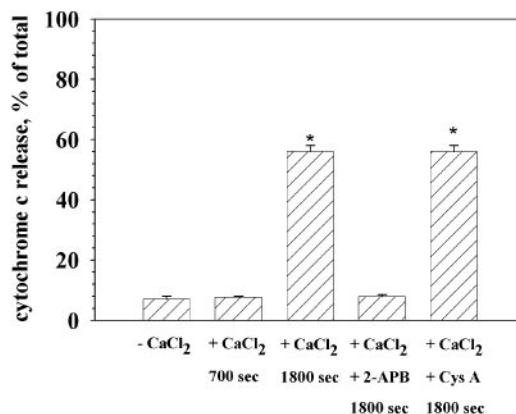


FIG. 5. Cytochrome *c* release (% of the total) estimated by enzyme-linked immunosorbent assay from mitochondria incubated in the presence or absence of 2-APB or cyclosporin A and challenged by high calcium loading.  $-\text{CaCl}_2$ , no calcium added;  $+\text{CaCl}_2$  700 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 700 s;  $+\text{CaCl}_2$  1800 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s;  $+\text{CaCl}_2$  + 2-APB 1800 s, 100  $\mu\text{M}$  2-APB was added at 50 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s;  $+\text{CaCl}_2$  + Cys A 1800 s, 2  $\mu\text{M}$  cyclosporin A (Cys A) was added at 50 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s. Samples were sedimented at 14,000  $\times g$  for 3 min, and both supernatants and pellets were probed for cytochrome *c*, \*, significant,  $p < 0.05$ , one-way analysis of variance, Dunnett's *post hoc* analysis,  $n = 4$ .

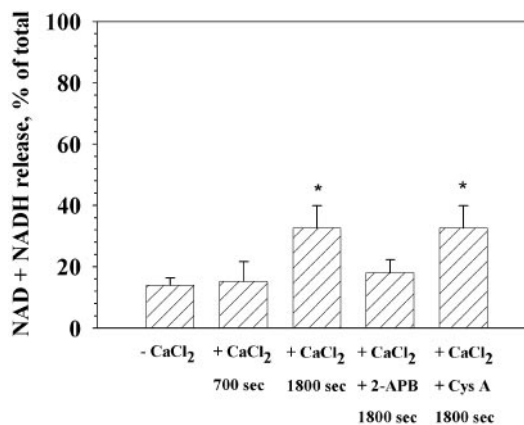


FIG. 6. NAD + NADH release (% of the total) estimated as described under “Experimental Procedures” from mitochondria incubated in the presence or absence of 2-APB or cyclosporin A and challenged by high calcium loading.  $-\text{CaCl}_2$ , no calcium added;  $+\text{CaCl}_2$  700 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 700 s;  $+\text{CaCl}_2$  1800 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s;  $+\text{CaCl}_2$  + 2-APB 1800 s, 100  $\mu\text{M}$  2-APB was added at 50 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s;  $+\text{CaCl}_2$  + Cys A 1800 s, 2  $\mu\text{M}$  cyclosporin A (Cys A) was added at 50 s, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s, and the sample was collected at 1800 s. Samples were sedimented at 14,000  $\times g$  for 3 min, and both supernatants and pellets were probed for pyridine nucleotides. \*, significant,  $p < 0.05$ , one-way analysis of variance, Dunnett's *post hoc* analysis,  $n = 4$ .

release was observed (Fig. 3). 2-APB but not cyclosporin A inhibited  $\text{Ca}^{2+}$ -induced cytochrome *c* release, consistent with the differences in the effects of these drugs observed on mCICR. The same pattern was observed for release of matrix pyridine nucleotides (Fig. 6). The appearance of pyridine nucleotides in the medium over that observed in the absence of  $\text{Ca}^{2+}$  occurred after the onset of mCICR and was inhibited by 2-APB but not cyclosporin A.

**Effects of 2-APB on Mitochondrial Bioenergetics and Relationship to Inhibition of mCICR**—The effects of 2-APB on res-

TABLE I  
Dose-dependent effect of 2-APB on oxygen consumption (nmol O<sub>2</sub>/min/mg protein), driven by NAD-linked (glutamate + malate) or FADH<sub>2</sub>-linked (succinate) substrates. NS, not significant.

Substrate	Glutamate (5 mM) + malate (5 mM)					Succinate (5 mM) + rotenone (1 μM)	
	Control	1 μM 2-APB	10 μM 2-APB	50 μM 2-APB	100 μM 2-APB	Control	100 μM 2-APB
State 3	137 ± 14	137 ± 13 (NS)	96 ± 2 <sup>a</sup>	79 ± 10 <sup>a</sup>	67 ± 3 <sup>a</sup>	141 ± 3	96 ± 2 <sup>b</sup>
State 4	15 ± 1	16 ± 2 (NS)	25 ± 1 <sup>a</sup>	27 ± 1 <sup>a</sup>	31 ± 2 <sup>a</sup>	35 ± 2	51 ± 1 <sup>b</sup>

<sup>a</sup> significant,  $p < 0.05$  one-way analysis of variance, Dunnett's *post hoc* analysis.

<sup>b</sup> significant,  $p < 0.001$ , Student's *t* test, ( $n = 4$ ).

piration by isolated non-synaptosomal brain mitochondria are described in Table I. 2-APB exhibited a dose-dependent inhibition of State 3 respiration with the NADH-linked oxidizable substrates malate plus glutamate, with significant inhibition observed at a concentration of 10 μM and a 57% inhibition observed at 100 μM. 2-APB similarly inhibited uncoupler (FCCP) stimulated respiration (not shown). State 4 respiration was significantly elevated at 10 μM 2-APB and was approximately twice the control rate at 100 μM. Qualitatively similar results were obtained with mitochondria respiring on succinate in the presence of rotenone (Table I). These results indicate that 2-APB is both a mild respiratory inhibitor and uncoupler at concentrations that protect against activation of the PTP.

The possibility that 2-APB protection against mCICR is related to its effects on respiration was tested by measuring mitochondrial Ca<sup>2+</sup> uptake and release in the presence of targeted respiratory inhibition. When NADH-linked respiration is completely inhibited by rotenone, active Ca<sup>2+</sup> uptake can still be driven by ΔΨ established via ATP-dependent H<sup>+</sup> export catalyzed by the mitochondrial F<sub>0</sub>-F<sub>1</sub>-ATPase (49). As shown in Fig. 7a, the onset of mCICR in the presence of rotenone occurred at a smaller Ca<sup>2+</sup> load (800 nmol) than that required in the absence of rotenone (1200 nmol; Fig. 2). Under this state of complete respiratory inhibition, 100 μM 2-APB still inhibited mCICR (Fig. 7a, *thick versus thin line*), indicating that its moderate inhibition of O<sub>2</sub> consumption is unrelated to its ability to inhibit the PTP. Ca<sup>2+</sup> uptake was slower in the presence of 2-APB, likely due to its moderate uncoupling effect.

The possible dependence of 2-APB protection against mCICR on mitochondrial ATP hydrolysis was tested by exposing mitochondria to Ca<sup>2+</sup> in the absence of rotenone but in the presence of the ATPase inhibitor oligomycin. Under this condition, mCICR was also activated by 800 nmol of Ca<sup>2+</sup> and inhibited by 2-APB (Fig. 7b). Ca<sup>2+</sup> influx was significantly slower in the presence of 2-APB, as expected by the inhibition of ATPase-dependent Ca<sup>2+</sup> uptake (50) together with the inhibition of respiration by 100 μM 2-APB. The observations that 2-APB is effective at inhibiting mCICR in the absence and the presence of either rotenone or oligomycin indicates that its mechanism is closely related to pore opening and not due to its moderate effects on mitochondrial bioenergetics. In addition to that, moderate uncoupling could prove beneficial to a mitochondrion against noxious stimuli, lowering its ability to form free radicals at a basal level (51). Therefore, FCCP concentration was titrated to confer the same extent of depolarization (30 nM, Fig. 7c, *curve a*) and activation of state 4 respiration (5 nM, Fig. 7c, *curve b*), and this range of concentration was tested as a possible protectant against PTP; under these conditions, mCICR was still present.

**Inhibition of Mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> Exchanger Does Not Reproduce the Effect of 2-APB**—Studies demonstrating 2-APB inhibition of mitochondrial Ca<sup>2+</sup> efflux after a capacitative Ca<sup>2+</sup> entry event hypothesized the involvement of the mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (32). Pretreatment of isolated brain mitochondria with the inhibitor of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger CGP 37157 (20 μM) in the absence of Na<sup>+</sup> did not inhibit mCICR and actually promoted the release of Ca<sup>2+</sup> (Fig.

8, *curve a*). Moreover, the simultaneous presence of 2-APB (100 μM) and CGP 37157 (20 μM) resulted in much slower Ca<sup>2+</sup> uptake and partial antagonism of the protective effect of 2-APB (Fig. 8, *curve c*; compare with Fig. 3, *curve d*). When medium K<sup>+</sup> was substituted for Na<sup>+</sup> at 10–40 mM, mCICR was unaffected and was not inhibited by CGP 37157 (not shown). It therefore appears that Na<sup>+</sup>/Ca<sup>2+</sup> exchange activity does not contribute to the mCICR observed in these experiments and is not a target of 2-APB.

**Comparison of 2-APB with Other PTP Inhibitors**—Experiments identical to that described by Fig. 2 performed in the presence of NADH-linked substrates and ATP were conducted in the presence of other compounds reported to inhibit the PTP or other related cellular activities. Results are expressed as the concentration of medium-free Ca<sup>2+</sup> present at 1800 s of mitochondrial incubation and after three additions of 400 nmol of Ca<sup>2+</sup>. Although the presence of 100 μM 2-APB allowed brain mitochondria to maintain the medium [Ca<sup>2+</sup>] at <1 μM, no compound other than bongkreic acid inhibited mCICR to an extent that allowed for maintenance of the medium [Ca<sup>2+</sup>] at <20 μM. These compounds included phospholipase A<sub>2</sub> inhibitors, *i.e.* trifluoperazine, bromoenol lactone, and aristolochic acid (52), (53, 54), BSA, which binds free fatty acids, antioxidants, *i.e.* superoxide dismutase, catalase, *N*-acetylcysteine, butylated hydroxytoluene, ubiquinone 0 (55), inhibitors of nitric-oxide synthesis, *i.e.* 7-nitroindazole and 1400 W (56), and other agents, *e.g.* spermine and tamoxifen (57, 58), which inhibit the PTP by other mechanisms. Xestospongine C, a potent inositol 1,4,5-triphosphate receptor blocker (59), was also tested due to the fact that it has been proposed to resemble the active, dimerized form of 2-APB (Ref. 60, but see also Dobryneva and Blackmore (61)). Because of the documented inhibitory effect of 2-APB on inositol 1,4,5-triphosphate receptors (30) and sarco-endoplasmic reticulum Ca<sup>2+</sup> ATPase pump (62), it was prudent to exclude the conception that the increase of Ca<sup>2+</sup> in the medium and its inhibition by 2-APB upon high calcium loading was attributed to contaminating microsomes and/or endoplasmic reticulum. The addition of inositol 1,4,5-triphosphate (100 μM) or thapsigargin (2 μM) upon completion of Ca<sup>2+</sup> uptake did not cause any acceleration of Ca<sup>2+</sup> release (not shown).

## DISCUSSION

The most important new information obtained from these experiments is the identification of 2-APB as an agent capable of inhibiting the PTP in brain mitochondria under physiologically relevant *in vitro* conditions in the presence of millimolar concentrations of ATP. The ability to block PTP in the presence of adenine nucleotides is also relevant to the potential involvement of PTP in ischemic brain injury. Even though ATP levels may fall to less than the millimolar range during ischemic deenergizing, ADP levels approximate 400 μM (63), and the *K<sub>i</sub>* for inhibition of the PTP by ADP is in the low micromolar range (64). Although both 2-APB and the classical PTP inhibitor cyclosporin A inhibit the PTP in the absence of adenine nucleotides (Fig. 1), neither cyclosporin A or a host of other agents that are reported to inhibit mitochondrial permeability transi-

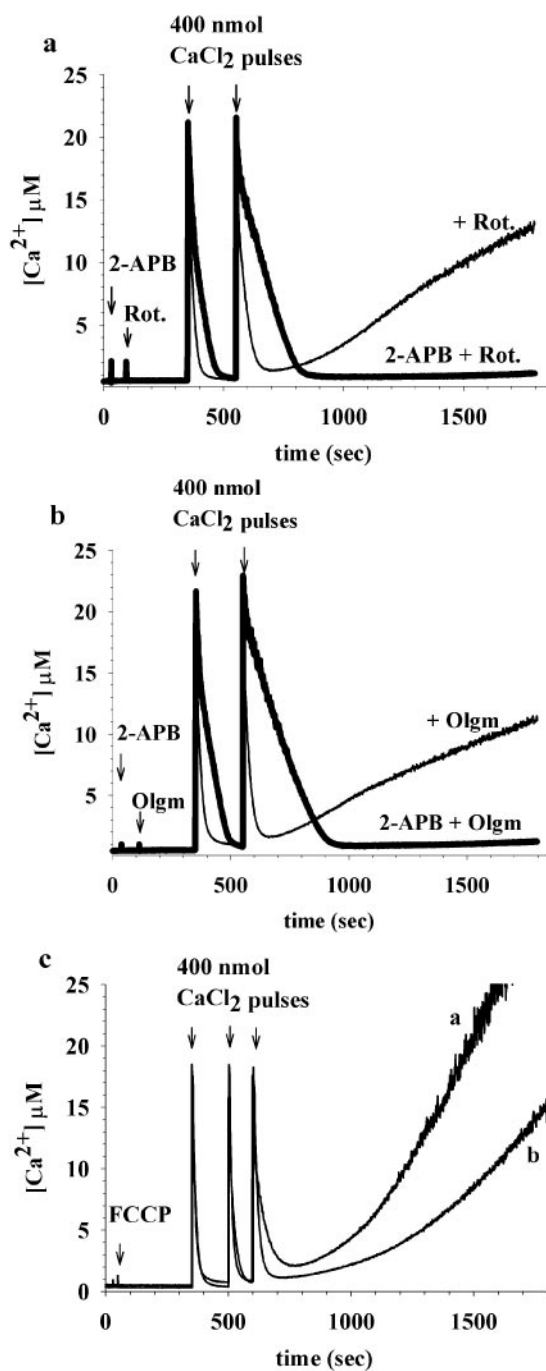


FIG. 7. *a*, effect of 2-APB on mCICR in the presence of rotenone ( $1 \mu\text{M}$ ) followed by Fura 6F fluorescence (calibrated data). 2-APB ( $100 \mu\text{M}$ ) was added at 50 s, rotenone (*Rot.*) was added at 100 s, and 400 nmol of  $\text{CaCl}_2$  was added at 350 and 550 s; *thick line*, mitochondria treated with 2-APB. The graph is typical and is one of three independent experiments. *b*, effect of 2-APB on mCICR in the presence of oligomycin ( $2 \mu\text{M}$ ) followed by Fura 6F fluorescence (calibrated data). 2-APB ( $100 \mu\text{M}$ ) was added at 50 s, oligomycin was added at 100 s, and 400 nmol of  $\text{CaCl}_2$  added at 350 and 550 s; *thick line*, mitochondria treated with 2-APB. The graph is typical and is one of three independent experiments. *c*, effect of moderate uncoupling by FCCP on mCICR followed by Fura 6F fluorescence (calibrated data). FCCP added at 50 s, and 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s. *Curve a*, 30 nM FCCP; *curve b*, 5 nM FCCP. The graph is typical and is one of three independent experiments.

tion under some conditions is capable of inhibiting the PTP in non-synaptosomal brain mitochondria in the presence of ATP (Fig. 2 and Table II). Inhibition of PTP by cyclosporin A is variable (27, 65), pointing to the possibility of the presence of a

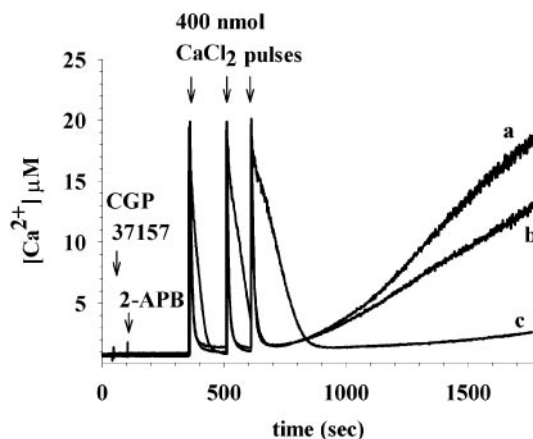


FIG. 8. Effect of CGP 37157 in the presence and absence of 2-APB on mCICR followed by Fura 6F fluorescence (calibrated data). *a*, CGP 37157 ( $20 \mu\text{M}$ ) was added at 50 s, and 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s. *b*, 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s. *c*, CGP 37157 ( $20 \mu\text{M}$ ) was added at 100 s, 2-APB was added at 100 s, and 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s. The graph is a typical and is one of three independent experiments.

TABLE II

2-APB effect upon high calcium loading of brain mitochondria and subsequent  $\text{Ca}^{2+}$  release versus previously identified PTP inhibitors. Mitochondria were pretreated with each compound at 50 s, then 400 nmol of  $\text{CaCl}_2$  was added at 350, 500, and 600 s

Inhibitor	$[\text{Ca}^{2+}]$ at 1800 s $\mu\text{M}$
No additions	$21.2 \pm 2.8$
2-APB ( $100 \mu\text{M}$ )	$0.64 \pm 0.12^a$
Cyclosporin A ( $1 \mu\text{M}$ )	$18.2 \pm 0.24$
Bongkreikic acid ( $20 \mu\text{M}$ )	$0.62 \pm 0.14^a$
Trifluoperazine ( $100 \mu\text{M}$ )	$20.2 \pm 2.4$
Bromo-enol lactone ( $10 \mu\text{M}$ )	$21.1 \pm 2.8$
Aristolochic acid ( $50 \mu\text{M}$ )	$20.8 \pm 2.0$
Bovine serum albumin (fatty acid-free) (0.1 %)	$22.2 \pm 2.4$
Spermine ( $100 \mu\text{M}$ )	$20.3 \pm 2.2$
7-Nitroindazole ( $100 \mu\text{M}$ )	$22.6 \pm 2.5$
1400W ( $100 \mu\text{M}$ )	$21.9 \pm 2.9$
Ubiquinone 0 ( $10 \mu\text{M}$ )	$20.2 \pm 2.7$
Butylated hydroxytoluene ( $100 \mu\text{M}$ )	$19.5 \pm 2.5$
<i>N</i> -Acetylcysteine (5 mM)	$22.2 \pm 2.1$
Catalase (100 units)	$21.1 \pm 2.3$
Superoxide dismutase (40 units)	$21.2 \pm 2.7$
Tamoxifen (0.3–10 $\mu\text{M}$ )	$20.1 \pm 2.1$
Xestospingon C ( $1 \mu\text{M}$ )	$22.3 \pm 2.3$

<sup>a</sup> Significant,  $p < 0.05$  one-way analysis of variance, Dunnett's *post hoc* analysis,  $n = 3$ .

more fundamental constituent of PTP other than the one conferring its cyclosporin A sensitivity. Such a constituent is exemplified by the effect of bongkreikic acid. Other than 2-APB, this compound is the only reported PTP inhibitor to block mCICR in brain mitochondria in the presence of ATP (Fig. 3 and Table II). Bongkreikic acid locks the adenine nucleotide translocator at the M configuration. This protein has been implicated as a component of a multiprotein complex that constitutes the PTP (8). Bongkreikic acid, at concentrations required to inhibit the PTP, completely inhibits State 3 respiration due to the inhibition of the adenine nucleotide translocator. 2-APB also inhibits respiration but to a much smaller extent (Table I). Because both ADP- and FCCP-stimulated respiration are affected by 2-APB, it likely affects the electron transport chain rather than adenine nucleotide transport.

Experiments were performed to determine whether the effects of 2-APB on respiration and  $\Delta\Psi$  are related to its PTP inhibitory activity. 2-APB inhibited mCICR in the presence of the respiratory inhibitor rotenone and in the presence of the ATP synthase inhibitor oligomycin (Fig. 7, *a* and *b*, respective-

ly). Moreover, mCICR was not inhibited by the protonophore FCCP at a concentration that mimics the respiratory uncoupling and mitochondrial membrane depolarization observed with 100  $\mu\text{M}$  2-APB (Fig. 7c). Therefore, the moderate effects of 2-APB on respiration are unrelated to its potent inhibition of the PTP. Although moderate respiratory inhibition by 2-APB is not responsible for PTP inhibition, it probably accounts for the slightly slower rate of  $\text{Ca}^{2+}$  uptake observed in the presence of 2-APB in the presence of ATP (Figs. 2 and 3). In the absence of ATP, however, 2-APB actually accelerated the rate of mitochondrial net  $\text{Ca}^{2+}$  uptake (Fig. 1b). In the absence of ATP, even small additions of  $\text{Ca}^{2+}$  induce the PTP in a significant fraction of the mitochondrial population (26), thereby reducing the number of mitochondria capable of energy-dependent  $\text{Ca}^{2+}$  accumulation. Thus, 2-APB accelerates  $\text{Ca}^{2+}$  uptake in the absence of ATP by maintaining a larger population of energized mitochondria.

The PTP can assume different conductance states, including that of a low conductance pore (4), that causes membrane depolarization but not osmotic lysis unless followed by transition to a high conductance permeability (66). Because the mCICR observed in our experiments was accompanied by release of cytochrome *c* and pyridine nucleotides at  $\sim 800$  s of incubation (Figs. 5 and 6, respectively), the high conductance pore was clearly activated at this time. The additional observation that  $\Delta\Psi$  was reduced by  $\text{Ca}^{2+}$  additions before release of pyridine nucleotides and cytochrome *c* supports the possibility that activation of the low conductance pore preceded that of the high conductance PTP (Fig. 4a). It is therefore possible that 2-APB inhibits the transition from the low to high conductance mode (66). The addition of ruthenium red (1  $\mu\text{M}$ ) or its purified active component Ru360 (165 nM) just before the onset of mCICR induced an abrupt release of  $\text{Ca}^{2+}$ , supporting the possibility of an induction of  $\text{Ca}^{2+}$  cycling across the mitochondrial membrane (18, 67), leading progressively to opening of the pore throughout the heterogeneous mitochondrial population (26).

Inhibition of the consequences of PTP activation on brain mitochondria by 2-APB provides a rationale for testing the potential neuroprotective actions of this compound. Despite the mild uncoupling effect of 2-APB, it protected against complete depolarization caused by mitochondrial  $\text{Ca}^{2+}$  overload (Fig. 4b) and, therefore, protects against  $\text{Ca}^{2+}$ -induced impairment of oxidative phosphorylation. 2-APB also prevented the loss of matrix pyridine nucleotides and cytochrome *c*, both also necessary for respiration and oxidative phosphorylation. Release of cytochrome *c* can stimulate mitochondrial production reactive oxygen species (68), and loss of pyridine nucleotides and other matrix compounds, e.g. glutathione, can compromise their detoxification. Thus, 2-APB may protect against  $\text{Ca}^{2+}$ -induced mitochondrial oxidative stress as well as metabolic failure.

The results of this investigation also relate to the contribution of mitochondrial  $\text{Ca}^{2+}$  transport to normal cellular  $\text{Ca}^{2+}$  homeostasis and signal transduction. Mitochondria within cells may form an electrically coupled network, synchronizing electrical signals generated by the PTP (69).  $\text{Ca}^{2+}$  plays a pivotal role in induction and priming of the PTP. Therefore, PTP inhibitors and other agents that modulate mitochondrial  $\text{Ca}^{2+}$  handling can be useful tools to elucidate the roles of mitochondria in  $\text{Ca}^{2+}$ -mediated signal transduction.

In addition to our demonstration that 2-APB is an effective blocker of PTP, this compound is a blocker of store-operated  $\text{Ca}^{2+}$  entry (70), a phenomenon recently identified to be centrally positioned among signal transduction and  $[\text{Ca}^{2+}]_i$  homeostasis in both excitable and non-excitable cells (71). Inhibition of SOCE by 2-APB was attributed to impairment of both ino-

sitol 1,4,5-triphosphate-mediated  $\text{Ca}^{2+}$  release (30) and inhibition of TRP channels (72), (73). However, TRP channels have yet to be unequivocally established as the molecular basis for SOCE (74). 2-APB also inhibits the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase pump (62) and voltage-dependent  $\text{K}^+$  channels (75) and blocks gap junctions, possibly by interfering with the docking interactions of two gap junctional hemi-channels (76).

Activation of the mitochondrial PTP may oppose SOCE, based on the fact that SOCE is inhibited by high  $[\text{Ca}^{2+}]_i$  and the observation that abolition of  $\text{Ca}^{2+}$  uptake by mitochondria inactivates SOCE (77–79). Thus, it is possible that release of mitochondrial  $\text{Ca}^{2+}$  stores through activation of the PTP elevate  $[\text{Ca}^{2+}]_i$ , thereby inhibiting SOCE. Interpretation of results obtained from SOCE measurements in the presence of 2-APB is, therefore, potentially complicated by the effect this agent has on mitochondrial  $\text{Ca}^{2+}$  handling.

Considering the reported effects of 2-APB on TRP channels, we attempted to identify immunoreactivity of TRP family members (TRPC1, -3, -4, -6) in highly purified mitochondrial subfractions (outer membrane, contact sites, inner membrane) but were unsuccessful (not shown). 2-APB may block the activation machinery of the channels participating in SOCE and not the channels themselves (80), (81). Therefore, 2-APB-sensitive channel-activating proteins similar to those involved in SOCE may be present in mitochondria in the absence of TRP channels. These proteins may interact with one or more of the many mitochondrial cation channels that have been identified electrophysiologically but not adequately characterized (82).

In conclusion, we show that 2-APB inhibits mCICR in isolated non-synaptosomal brain mitochondria in the presence of adenine nucleotides and magnesium. Neither inhibition of the reversal of the uniporter nor blockade of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger could reproduce the effect of 2-APB.  $\text{Ca}^{2+}$  release was accompanied by cyclosporin A-insensitive loss of cytochrome *c* and pyridine nucleotide release, signifying activation of the "high conductance" pore. These observations taken together with others demonstrating inhibition of SOCE by 2-APB indicate a mitochondrial site of action for this drug in addition to other cell membranes.

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#### REFERENCES

- Csordas, G., Thomas, A. P., and Hajnoczky, G. (1999) *EMBO J.* **18**, 96–108
- Simpson, P. B., and Russell, J. T. (1996) *J. Biol. Chem.* **271**, 33493–33501
- Evtodienco, Y., Teplova, V., Khawaja, J., and Saris, N. E. (1994) *Cell Calcium* **15**, 143–152
- Ichas, F., Jouaville, L. S., and Mazat, J. P. (1997) *Cell* **89**, 1145–1153
- Montero, M., Alonso, M. T., Albillos, A., Garcia-Sancho, J., and Alvarez, J. (2001) *Mol. Biol. Cell* **12**, 63–71
- Hunter, D. R., and Haworth, R. A. (1979) *Arch. Biochem. Biophys.* **195**, 468–477
- Haworth, R. A., and Hunter, D. R. (1979) *Arch. Biochem. Biophys.* **195**, 460–467
- Hunter, D. R., and Haworth, R. A. (1979) *Arch. Biochem. Biophys.* **195**, 453–459
- Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta* **1241**, 139–176
- Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. (1998) *Biochim. Biophys. Acta* **1366**, 177–196
- Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786
- Ichas, F., Jouaville, L. S., Sidash, S. S., Mazat, J. P., and Holmuhamedov, E. L. (1994) *FEBS Lett.* **348**, 211–215
- Minamikawa, T., Williams, D. A., Bowser, D. N., and Nagley, P. (1999) *Exp. Cell Res.* **246**, 26–37
- Fournier, N., Ducet, G., and Crevat, A. (1987) *J. Bioenerg. Biomembr.* **19**, 297–303
- Di Lisa, F., Menabo, R., Canton, M., Barile, M., and Bernardi, P. (2001) *J. Biol. Chem.* **276**, 2571–2575
- Huser, J., Rechenmacher, C. E., and Blatter, L. A. (1998) *Biophys. J.* **74**, 2129–2137
- Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., and Herman, B. (1997) *Mol. Cell. Biochem.* **174**, 159–165
- Kristal, B. S., and Dubinsky, J. M. (1997) *J. Neurochem.* **69**, 524–538
- He, L., and Lemasters, J. J. (2002) *FEBS Lett.* **512**, 1–7

20. Brustovetsky, N., and Dubinsky, J. M. (2000) *J. Neurosci.* **20**, 8229–8237
21. Brustovetsky, N., and Dubinsky, J. M. (2000) *J. Neurosci.* **20**, 103–113
22. Malkevitch, N. V., Dedukhova, V. I., Simonian, R. A., Skulachev, V. P., and Starkov, A. A. (1997) *FEBS Lett.* **412**, 173–178
23. Novgorodov, S. A., Gudiz, T. I., Milgrom, Y. M., and Brierley, G. P. (1992) *J. Biol. Chem.* **267**, 16274–16282
24. Novgorodov, S. A., Gudiz, T. I., Brierley, G. P., and Pfeiffer, D. R. (1994) *Arch. Biochem. Biophys.* **311**, 219–228
25. Andreyev, A., and Fiskum, G. (1999) *Cell Death Differ.* **6**, 825–832
26. Kristian, T., Weatherby, T. M., Bates, T. E., and Fiskum, G. (2002) *J. Neurochem.* **83**, 1297–1308
27. Kobayashi, T., Kuroda, S., Tada, M., Houkin, K., Iwasaki, Y., and Abe, H. (2003) *Brain Res.* **960**, 62–70
28. Berman, S. B., Watkins, S. C., and Hastings, T. G. (2000) *Exp. Neurol.* **164**, 415–425
29. Brustovetsky, N., Brustovetsky, T., Jemmerson, R., and Dubinsky, J. M. (2002) *J. Neurochem.* **80**, 207–218
30. Maruyama, T., Kanaji, T., Nakade, S., Kanno, T., and Mikoshiba, K. (1997) *J. Biochem. (Tokyo)* **122**, 498–505
31. Prakriya, M., and Lewis, R. S. (2001) *J. Physiol.* **536**, 3–19
32. Gunter, T. E., Buntinas, L., Sparagna, G., Eliseev, R., and Gunter, K. (2000) *Cell Calcium* **28**, 285–296
33. Sims, N. R. (1990) *J. Neurochem.* **55**, 698–707
34. Bers, D. M., Patton, C. W., and Nuccitelli, R. (1994) *Methods Cell Biol.* **40**, 3–29
35. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
36. Klingenberg, M. (1985) *Methods of Enzymatic Analysis*, 3rd Ed., pp. 251–269, VCH Publishers, Inc., Deerfield Beach, FL
37. Sparagna, G. C., Gunter, K. K., Sheu, S. S., and Gunter, T. E. (1995) *J. Biol. Chem.* **270**, 27510–27515
38. Stout, A. K., and Reynolds, I. J. (1999) *Neuroscience* **89**, 91–100
39. Neher, E. (1998) *Neuron* **20**, 389–399
40. Szalai, G., Csordas, G., Hantash, B. M., Thomas, A. P., and Hajnoczky, G. (2000) *J. Biol. Chem.* **275**, 15305–15313
41. Montero, M., Alonso, M. T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., Garcia, A. G., Garcia-Sancho, J., and Alvarez, J. (2000) *Nat. Cell Biol.* **2**, 57–61
42. Dykens, J. A., and Stout, A. K. (2001) *Methods Cell Biol.* **65**, 285–309
43. Valle, V. G., Pereira-da-Silva, L., and Vercesi, A. E. (1986) *Biochem. Biophys. Res. Commun.* **135**, 189–195
44. Scaduto, R. C., Jr., and Grotyohann, L. W. (1999) *Biophys. J.* **76**, 469–477
45. Crompton, M. (2000) *J. Physiol.* **529**, 11–21
46. Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. (1999) *Eur. J. Biochem.* **264**, 687–701
47. Appaix, F., Guerrero, K., Rampal, D., Izikki, M., Kaambre, T., Sikk, P., Brdiczka, D., Riva-Lavieille, C., Olivares, J., Longuet, M., Antonsson, B., and Saks, V. A. (2002) *Biochim. Biophys. Acta* **1556**, 155–167
48. Kushnareva, Y. E., and Sokolove, P. M. (2000) *Arch. Biochem. Biophys.* **376**, 377–388
49. Gunter, T. E., Gunter, K. K., Sheu, S. S., and Gavin, C. E. (1994) *Am. J. Physiol.* **267**, C313–C339
50. Tjioe, S., Bianchi, C. P., and Haugaard, N. (1970) *Biochim. Biophys. Acta* **216**, 270–273
51. Skulachev, V. P. (1996) *FEBS Lett.* **397**, 7–10
52. Broekemeier, K. M., and Pfeiffer, D. R. (1995) *Biochemistry* **34**, 16440–16449
53. Broekemeier, K. M., Iben, J. R., LeVan, E. G., Crouser, E. D., and Pfeiffer, D. R. (2002) *Biochemistry* **41**, 7771–7780
54. Pastorino, J. G., Simbula, G., Yamamoto, K., Glascott, P. A., Jr., Rothman, R. J., and Farber, J. L. (1996) *J. Biol. Chem.* **271**, 29792–29798
55. Kowaltowski, A. J., Castilho, R. F., and Vercesi, A. E. (2001) *FEBS Lett.* **495**, 12–15
56. Horn, T. F., Wolf, G., Duffy, S., Weiss, S., Keilhoff, G., and MacVicar, B. A. (2002) *FASEB J.* **16**, 1611–1622
57. Custodio, J. B., Moreno, A. J., and Wallace, K. B. (1998) *Toxicol. Appl. Pharmacol.* **152**, 10–17
58. Lapidus, R. G., and Sokolove, P. M. (1992) *FEBS Lett.* **313**, 314–318
59. Gaffni, J., Munsch, J. A., Lam, T. H., Catlin, M. C., Costa, L. G., Molinski, T. F., and Pessah, I. N. (1997) *Neuron* **19**, 723–733
60. van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2000) *J. Biol. Chem.* **275**, 28562–28568
61. Dobrydneva, Y., and Blackmore, P. (2001) *Mol. Pharmacol.* **60**, 541–552
62. Bilmen, J. G., Wootton, L. L., Godfrey, R. E., Smart, O. S., and Michelangeli, F. (2002) *Eur. J. Biochem.* **269**, 3678–3687
63. Ekholm, A., Katsura, K., Kristian, T., Liu, M., Folbergrova, J., and Siesjö, B. K. (1993) *Brain Res.* **604**, 185–191
64. Haworth, R. A., and Hunter, D. R. (1980) *J. Membr. Biol.* **54**, 231–236
65. Hansson, M. J., Persson, T., Friberg, H., Keep, M. F., Rees, A., Wieloch, T., and Elmer, E. (2003) *Brain Res.* **960**, 99–111
66. Ichas, F., and Mazat, J. P. (1998) *Biochim. Biophys. Acta.* **1366**, 33–50
67. Riley, W. W., Jr., and Pfeiffer, D. R. (1985) *J. Biol. Chem.* **260**, 12416–12425
68. Starkov, A. A., Polster, B. M., and Fiskum, G. (2002) *J. Neurochem.* **83**, 220–228
69. De Giorgi, F., Lartigue, L., and Ichas, F. (2000) *Cell Calcium* **28**, 365–370
70. Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge, M. J., and Peppiatt, C. M. (2002) *FASEB J.* **16**, 1145–1150
71. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2002) *Nat. Cell Biol.* **4**, 263–272
72. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) *Cell* **108**, 595–598
73. Ma, H. T., Venkatachalam, K., Li, H. S., Montell, C., Kurotaki, T., Patterson, R. L., and Gill, D. L. (2001) *J. Biol. Chem.* **276**, 18888–18896
74. Zitt, C., Halaszovich, C. R., and Luckhoff, A. (2002) *Prog. Neurobiol.* **66**, 243–264
75. Wang, Y., Deshpande, M., and Payne, R. (2002) *Cell Calcium* **32**, 209–216
76. Harks, E. G., Camina, J. P., Peters, P. H., Ypey, D. L., Scheenen, W. J., Van Zoelen, E. J., and Theuvsen, A. P. (2003) *FASEB J.* **17**, 941–943
77. Hoth, M., Button, D. C., and Lewis, R. S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 10607–10612
78. Gilibert, J. A., and Parekh, A. B. (2000) *EMBO J.* **19**, 6401–6407
79. Hoth, M., Fanger, C. M., and Lewis, R. S. (1997) *J. Cell Biol.* **137**, 633–648
80. Ma, H. T., Venkatachalam, K., Parys, J. B., and Gill, D. L. (2002) *J. Biol. Chem.* **277**, 6915–6922
81. Schindl, R., Kahr, H., Graz, I., Groschner, K., and Romanin, C. (2002) *J. Biol. Chem.* **277**, 26950–26958
82. Antonenko, Y. N., Kinnally, K. W., and Tedeschi, H. (1991) *J. Membr. Biol.* **124**, 151–158