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Trifluoperazine and dibucaine-induced inhibition of glutamate-induced mitochondrial depolarization in rat cultured forebrain neurones

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1 Glutamate receptor activation has been previously shown to result in mitochondrial depolarization and activation of the mitochondrial permeability transition pore in cultured neurones. In this study, we characterized the effects of two putative permeability transition inhibitors, namely trifluoperazine and dibucaine, on mitochondrial depolarization in rat intact, cultured forebrain neurones.

2 Permeability transition was monitored by following mitochondrial depolarization in neurones loaded with the mitochondrial membrane potential-sensitive fluorescent indicator, JC-1. Trifluoperazine (10–20 μM) and dibucaine (50–100 μM) inhibited or delayed the onset of glutamate-induced permeability transition.

3 We also investigated the effects of trifluoperazine and dibucaine on neuronal recovery from glutamate-induced Ca^{2+} loads. Trifluoperazine affected Ca^{2+} recovery in a manner similar to the mitochondrial Na^+/Ca^{2+} exchange inhibitor, CGP-37157, while dibucaine had no apparent effect on Ca^{2+} recovery. Therefore, inhibition of permeability transition does not appear to be involved in Ca^{2+} recovery from glutamate-induced Ca^{2+} loads.

4 Trifluoperazine and dibucaine did not inhibit [^{3}H]-dizocilpine binding at the concentrations that prevented mitochondrial depolarization.

5 These studies suggest that trifluoperazine and dibucaine inhibit permeability transition in intact neurones. Trifluoperazine also appears to inhibit mitochondrial Na^+/Ca^{2+} exchange. These drugs should prove to be valuable tools in the further study of the role of mitochondrial permeability transition in glutamate-induced neuronal death.

Keywords: Permeability transition pore; mitochondria; sodium calcium exchange; trifluoperazine; dibucaine; glutamate; intracellular free calcium; JC-1; indo; CGP-37157

Introduction

Neurones are vulnerable to excessive concentrations of glutamate, and this cell death is dependent on increases in $[Ca^{2+}]_i$ (Michaels & Rothman, 1990; Hartley *et al*., 1993). This glutamate-mediated excitotoxicity has been implicated in the neurone loss incurred after stroke and head trauma (Choi, 1988) and can be easily modelled in neuronal culture (Rothman *et al*., 1987). Exactly which cellular death processes are specifically activated by excessive glutamate-triggered $[Ca^{2+}]_i$ entry have been the subject of intense study. Among the candidates for the Ca^{2+}-dependent mediators of glutamate toxicity that are stimulated specifically by NMDA receptor activation are reactive oxygen species generation (Dugan *et al*., 1995; Reynolds & Hastings, 1995; Bindokas *et al*., 1996), nitric oxide formation (Dawson *et al*., 1991) and increases in intracellular Mg^{2+} (Brocard *et al*., 1993; Hartnett *et al*., 1997).

Recently, several studies have suggested that activation of the mitochondrial permeability transition pore (PTP) occurs upon glutamate (*N*-methyl-D-aspartate; NMDA) receptor activation (Ankarcrona *et al*., 1996; Khodorov *et al*., 1996; Nieminen *et al*., 1996; Schinder *et al*., 1996; White & Reynolds, 1996). These studies found that cyclosporin A inhibited glutamate-induced mitochondrial membrane depolarization, which is the main evidence linking depolarization to PTP activation (Bernardi *et al*., 1994). The mitochondrial PTP, located in the inner membrane, when activated allows molecules with a molecular weight of less than 1.5 kD to pass freely into and out of the mitochondria (Zoratti & Szabo, 1995). Opening of this pore necessarily results in mitochondrial membrane depolarization. Activation of the PTP is toxic in many cell types (Griffiths & Halestrap, 1995; Nieminen *et al*., 1995). The PTP opens in response to excessive intramitochondrial Ca^{2+} and is also sensitive to increased oxidant levels (Zoratti & Szabo, 1995), both of which occur upon glutamate stimulation. Cyclosporin A also inhibits glutamate-induced neuronal death (Dawson *et al*., 1993; Schinder *et al*., 1996; White & Reynolds, 1996).

Mitochondria are important buffers of intracellular $[Ca^{2+}]_i$ increases caused by glutamate receptor activation (Wang *et al*., 1994; Kiedrowski & Costa, 1995; White & Reynolds, 1995). Upon withdrawal of glutamate, $[Ca^{2+}]_i$ decreases at a rate which is dependent on Ca^{2+} release from the Ca^{2+} loaded mitochondria. The mitochondrial Na/Ca^{2+} exchanger is the primary mechanism by which Ca^{2+} leaves the mitochondria under these conditions (Wang & Thayer, 1996; White & Reynolds, 1997). A specific inhibitor of this exchanger, CGP-37157 (Cox *et al*., 1993), causes a rapid fall in the $[Ca^{2+}]_i$ after glutamate removal (White & Reynolds, 1997). This suggests that the mitochondria are retaining Ca^{2+}, and that cytoplasmic Ca^{2+} buffering systems are able to clear Ca^{2+} efficiently out of the cytoplasm when the mitochondria are not adding to the cytoplasmic Ca^{2+} burden. When CGP-37157 is subsequently removed, cytoplasmic Ca^{2+} actually increases as a reflection of resumption of Ca^{2+} egress from the mitochondria.

Since mitochondrial dysfunction is a promising central target for the detrimental effects of glutamate we have investigated the effects of two drugs, trifluoperazine and dibucaine, which block PTP via a mechanism distinct from cyclosporin A (Broekemeier *et al*., 1989), on glutamate-induced mitochondrial depolarization in cultured forebrain neurones. We also tested whether these drugs affected Ca^{2+} buffering after a

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glutamate stimulus. These data further corroborate the findings of our laboratory (White & Reynolds, 1996) and others (Ankarcrona *et al*., 1996; Khodorov *et al*., 1996; Nieminen *et al*., 1996; Schinder *et al*., 1996) that PTP is activated as a result of glutamate receptor activation, and adds to the collection of useful tools for the further study of the role of mitochondria in glutamate toxicity.

Methods

Neuronal culture

Primary cultures of forebrain neurones were established from embryonic day 17 Sprague-Dawley rats as described previously (White & Reynolds, 1995). All procedures with the animals were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Use Committee of the University of Pittsburgh. Pregnant female rats were anaesthetized by ether inhalation until consciousness was lost, then were killed by decapitation. The pups were removed and also killed by decapitation. Forebrains were dissociated and plated in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and 24 u ml^{-1} penicillin, 24 $\mu g\ ml^{-1}$ streptomycin at a density of 3×10^5 cells ml^{-1} on 31 mm glass coverslips. One day after plating, the plating media was replaced with DMEM containing horse serum instead of foetal bovine serum and the coverslips were inverted to suppress proliferation of non-neuronal cells. Cultures were maintained at 37°C in a 5% CO_2 incubator until use (12–18 days). On the day of experiments, coverslips were rinsed with a HEPES buffered salt solution (HBSS) which contained (mM): NaCl 137, KCl 5, $MgSO_4$ 0.9. $CaCl_2$ 1.4, $NaHCO_3$ 3, Na_2HPO_4 0.6, KH_2PO_4 0.4, glucose 5.6 and HEPES 20, pH adjusted to 7.4 with NaOH, and fluorescent probes were loaded into the neurones as described below.

Mitochondrial membrane potential microfluorimetry

Relative measurements of mitochondrial membrane potential were made in individual neurones loaded with the ratiometric potential-sensitive fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (3 μM for 20 min at 37°C, followed by rinsing with HBSS and an additional 20 min at room temperature), as described previously (White & Reynolds, 1996) with an ACAS 570c confocal laser scanning microscope (Meridian Instruments, Okemos, MI). JC-1 monomers aggregate as a function of the mitochondrial membrane potential (Reers *et al*., 1991). JC-1 monomer fluorescence was monitored at 530 ± 10 nm, while JC-1 aggregate fluorescence was simultaneously monitored at 590 ± 17 nm following excitation at 488 nm. The ratio of aggregate fluorescence to monomer fluorescence is then a convenient index of mitochondrial membrane potential. JC-1 ratios were normalized to the starting (baseline) ratio for each neurone.

$[Ca^{2+}]_i$ microfluorimetry

$[Ca^{2+}]_i$ was measured in single neurones loaded with the calcium-sensitive fluorescent dye, indo-1 (White & Reynolds, 1995). On the day of the experiment, the cells were rinsed with HBSS and then incubated in HBSS containing 5 μM indo-1-AM and 5 $mg\ ml^{-1}$ bovine serum albumin, at 37°C for 50 min. After incubation the coverslip was rinsed with HBSS and returned to the incubator for an additional 20 min. The coverslip was then placed in a recording chamber (volume = 1 ml) and mounted on the stage of a Nikon Diaphot fluorescence microscope. Single cell indo-1 fluorescence was measured by isolating a neurone by use of a rectangular diaphragm that restricted the amount of fluorescent signal recorded. Solutions were perfused into the recording chamber at a rate of 20 $ml\ min^{-1}$. $[Ca^{2+}]_i$ was calculated from the ratios of indo-1 fluorescence obtained at 405 nm and 490 nm (excitation = 340 nm). *In situ* calibration parameters were used to convert ratios to $[Ca^{2+}]_i$ values, as described in detail previously (White & Reynolds, 1997).

[^{3}H]-dizocilpine binding

Ligand binding assays were performed in well-washed rat brain membranes as previously described (Reynolds & Palmer, 1991). Assays were performed in a final volume of 1 ml of 10 mM HEPES-NaOH (pH 7.4) that contained 0.2 mg protein, 0.5 nM [^{3}H]-dizocilpine, 100 μM glutamate, 30 μM glycine, and drugs (trifluoperazine or dibucaine) as appropriate at concentrations ranging from 1 μM to 500 μM. Non-specific binding was determined by 30 μM dizocilpine. Assays were incubated for 2 h at room temperature and terminated by filtration (3 rinses) over #32 glass-fibre filters (Schleicher and Schuell, Keene, NH) by use of a 24-well cells harvester (Brandel Inc.). Radioactivity was measured by a liquid scintillation counter at an efficiency of about 63%.

Materials

JC-1 and indo-1-AM were purchased from Molecular Probes (Eugene, OR). DMEM, MEM, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY). Glutamate, glycine, trifluoperazine and dibucaine were purchased from Sigma (St. Louis, MO). Dizocilpine was purchased from RBI (Natik, MA). [^{3}H]-dizocilpine was obtained from DuPont/NEN (Boston, MA). CGP-37157 (7-chloro-3,5-dihydro-5-phenyl–1H-4,1-benzothiazepine-2-on) was a generous gift from Ciba-Geigy Pharmaceuticals (Basel, Switzerland).

Results

The fluorescent ratiometric mitochondrial membrane potential indicator, JC-1, was used to measure mitochondrial membrane potential in cultured forebrain neurones. Glutamate (100 μM) resulted in a decrease in the JC-1 ratio (Figure 1a) which presumably represents depolarization of mitochondria. For comparison, a mitochondrial uncoupler, FCCP (750 nM), depolarized mitochondria to a normalized JC-1 ratio of 0.4 (data not shown) while nigericin caused mitochondrial hyperpolarization to a normalized ratio of 1.15 (White & Reynolds, 1996). In Figure 1b, the effect of trifluoperazine (20 μM) on this glutamate-induced depolarization can be seen. Trifluoperazine did not have a uniform effect on mitochondrial membrane potential. However, trifluoperazine added in the presence of glutamate did cause an overall delay or inhibition of glutamate-induced mitochondrial membrane depolarizaton, and even hyperpolarization in some neurones (Figures 1b and c). Solution change controls (no glutamate or trifluoperazine) did not alter the JC-1 signal for the duration of the recording. When added to intracellular solution containing 3 μM JC-1, trifluoperazine (20 μM) did not alter the aggregate (590 nm emission) fluorescence, suggesting that trifluoperazine does not directly alter JC-1 aggregation (data not shown). A summary of the effect of trifluoperazine alone and in combination with glutamate, is shown in Figure 1c. Trifluoperazine (10 or 20 μM) alone caused an increase in the neuronal JC-1 ratio, indicating mitochondrial membrane potential hyperpolarization. A concentration-dependent decrease in glutamate-induced mitochondrial membrane depolarization in the presence of trifluoperazine was evident. Thus, while the mitochondria did eventually depolarize in the presence of trifluoperazine, the onset of depolarization was delayed and the extent of depolarization was lessened by trifluoperazine (Figure 1c).

Dibucaine (50 or 100 μM) also modified glutamate-induced mitochondrial depolarization (Figure 2a). Dibucaine caused, in general, a delay in the onset of mitochondrial depolarization caused by glutamate and in some cells resulted in an initial hyperpolarization. The inhibitory effects of dibucaine on glu-

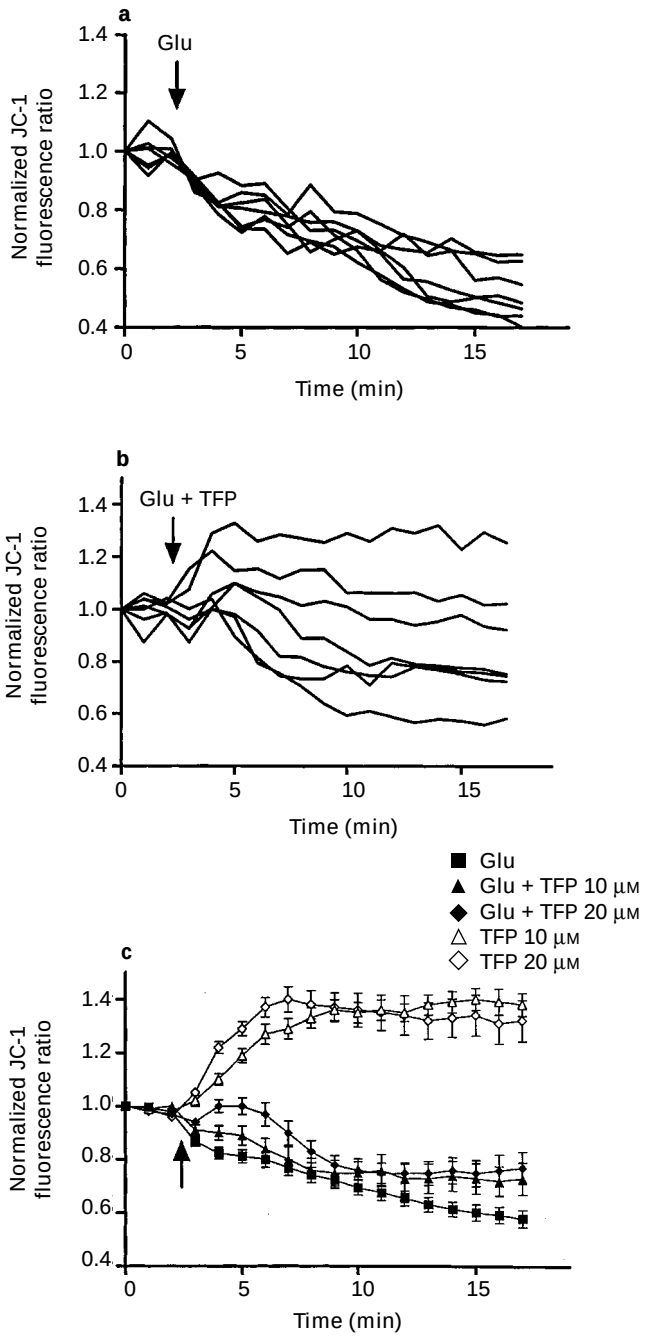

Figure 1 Effects of trifluoperazine on glutamate-induced mitochondrial membrane depolarization measured in intact cultured forebrain neurones. Fluorescence images of JC-1-loaded neurones were acquired with 488 nm excitation, and the ratio of images collected at 530±10 nm and 590±17 emission were normalized to the starting (untreated) ratio. An image was collected once every minute. Decreases in the JC-1 fluorescence ratio indicate mitochondrial membrane depolarization, while ratio increases indicate hyperpolarization. (a) Glutamate (Glu) cause mitochondrial depolarization. Representative simultaneous traces from 7 neurones exposed to 100 μM glutamate/1 μM glycine (arrow) after 3 min of baseline collection. The glutamate solution remained in contact with the neurones for the duration of the experiment (15 min). Methanol (0.05%) was included with the glutamate as a vehicle control. (b) Neurones were exposed 100 μM glutamate/1 μM glycine in the presence of 20 μM trifluoperazine (TFP) (arrow) for 15 min in these representative traces from 7 neurones recorded from simultaneously. (c) Summary of effects of trifluoperazine alone and in combination with 100 μM glutamate/1 μM glycine. The following treatments: 100 μM glutamate/10 μM trifluoperazine, 100 μM glutamate/20 μM trifluoperazine, 10 μM trifluoperazine and 20 μM trifluoperazine, were added at the arrow and remained in contact with the neurones for the rest of the experiment. Data points represent the mean of 32–56 neurones per condition from at least 3 different culture dates; vertical lines show s.e.mean.

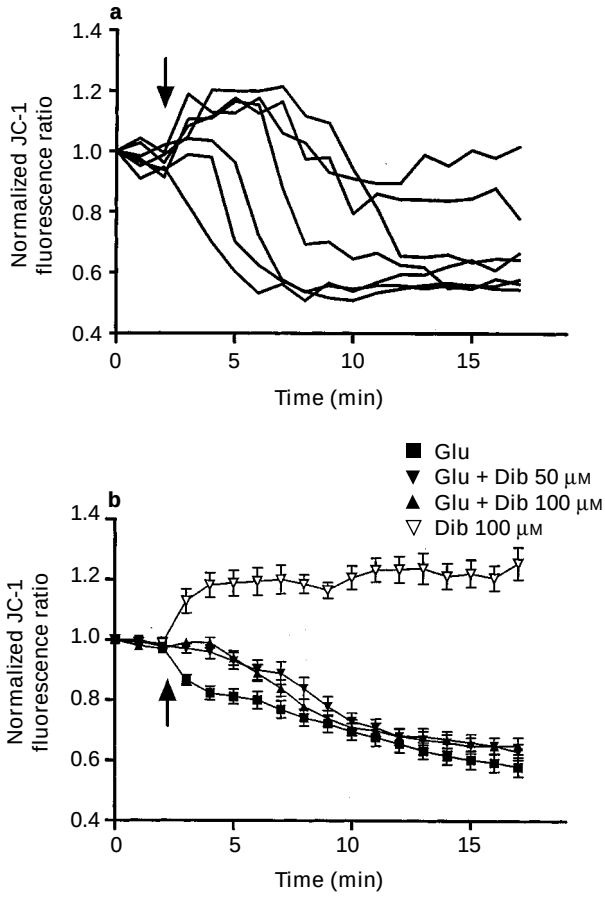

Figure 2 Effects of dibucaine (Dib) on glutamate-induced mitochondrial depolarization. Data were collected as in Figure 1. (a) Glutamate (100 μM) and glycine (1 μM) together with 100 μM dibucaine were added at the arrow. (b) Summary of effects of dibucaine alone and in combination with glutamate on mitochondrial membrane potential (100 μM glutamate, 100 μM glutamate/50 μM dibucaine, 100 μM glutamate/100 μM dibucaine, 100 μM dibucaine). Data points represent the mean of 37–59 neurones per condition from at least 3 different culture dates; vertical lines show s.e.mean.

tamate-induced mitochondrial membrane depolarization are summarized in Figure 2b. Interestingly, dibucaine alone, like trifluoperazine, caused mitochondrial hyperpolarization (Figure 2c). Dibucaine (100 μM) did not directly interfere with JC-1 aggregation *in vitro* (data not shown).

To rule out a direct inhibitory effect of trifluoperazine or dibucaine on NMDA receptor activation, we tested these drugs in a [^{3}H]-dizocilpine radioligand binding assay. [^{3}H]-dizocilpine specifically binds within the channel of activated NMDA receptors and is a convenient assay of NMDA receptor activation (Reynolds *et al*., 1987). Figure 3 shows that these drugs had minimal inhibitory effects on NMDA receptor activation at the concentrations used to inhibit PTP (i.e. 20 μM for trifluoperazine and 50 μM for dibucaine). Therefore, it is unlikely that the inhibitory effect of these drugs on glutamate-induced mitochondrial membrane depolarization reflects an inhibition of NMDA receptors, although this is clearly a property of these drugs at higher concentrations. As predicted from the binding results, dibucaine (100 μM) did not inhibit glutamate-induced Ca^{2+} increases in indo-1 loaded neurones (data not shown), excluding the possibility of a direct inhibitory action on the NMDA receptor or an indirect effect via inhibition of voltage-sensitive Na^+ channels (a prominent effect of dibucaine).

We have previously observed that inhibition of mitochondrial Na^+/Ca^{2+} exchange results in mitochondrial hyperpolarization (White & Reynolds, 1996). We investigated the effect of trifluoperazine and dibucaine on the rate of recovery from a

glutamate-induced $[Ca^{2+}]_i$ load as a marker of mitochondrial Na^+/Ca^{2+} exchange. In the stimulation paradigm exemplified in Figure 4a, indo-1-loaded neurones were first stimulated with a short (15 s) pulse of a low concentration of glutamate (3 μM)/glycine (1 μM) as a control. Fifteen minutes later, an intense glutamate (100 μM for 5 min) stimulus was applied, and the glutamate was washed out with Ca^{2+} free HBSS that contained methanol (control) (Figure 4a), CGP-37157 (Figure 4b), trifluoperazine (Figure 4c) or dibucaine (Figure 4d). These drugs were only present for the 2 min immediately following glutamate removal. After 2 min exposure to the drugs, the cells were continuously perfused with Ca^{2+} free HBSS until the experiment was terminated. Following 5 min of 100 μM glutamate, $[Ca^{2+}]_i$ recovered to baseline rather slowly (Figure 4a). The slow recovery appears to be due to the release of the mitochondrial Ca^{2+} pool because the introduction of a mitochondrial Na^+/Ca^{2+} inhibitor, CGP-37157, at the termination of the glutamate stimulus resulted in a rapid drop in $[Ca^{2+}]_i$ to near basal levels, followed by a secondary rise upon removal of the inhibitor (Figure 4b). Trifluoperazine modified the recovery from glutamate in a manner indistinguishable from that of CGP-37157 (Figure 4c), indicating the induction of a pronounced secondary rise when trifluoperazine was washed out. In contrast, dibucaine did not modify recovery (Figure 4d). Neither trifluoperazine nor dibucaine directly affected indo-1 fluorescence or intracellular Ca^{2+} in resting neurones (data not shown), thus excluding a possible effect of these drugs directly on the dye.

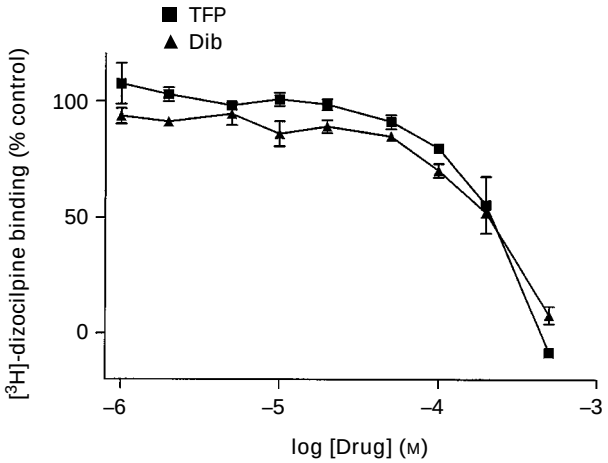

Figure 3 Effects of trifluoperazine (TFP) and dibucaine (Dib) on [^{3}H]-dizocilpine binding in rat brain homogenates. [^{3}H]-dizocilpine binding (in the presence of glutamate and glycine) is a measure of NMDA receptor activation. Substantial inhibition of binding was not evident until concentrations of trifluoperazine (or dibucaine) reached at least 100 μM. The IC_{50} values calculated for trifluoperazine and dibucaine were 197 μM and 209 μM, respectively. Data points represent the mean of 3 separate experiments; vertical lines show s.e.mean.

Discussion

Recent studies have identified a potential role for the activation of mitochondrial PTP in the excitotoxicity resulting from overactivation of glutamate receptors (Ankarcrona *et al*., 1996; Khodorov *et al*., 1996; Nieminen *et al*., 1996; Schinder *et al*.,

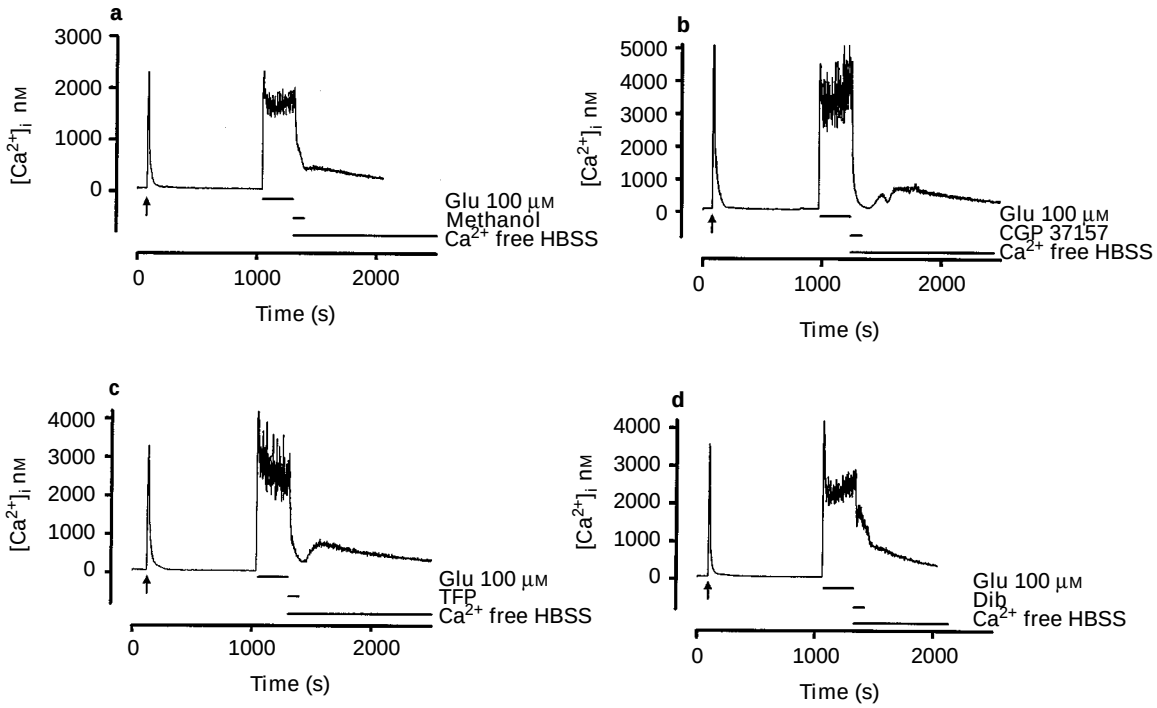

Figure 4 Effects of trifluoperazine (TFP) and dibucaine (Dib) on the recovery from glutamate-induced Ca^{2+} loads. Cultured rat forebrain neurones, loaded with indo-1, were exposed to a short pulse of 3 μM glutamate/1 μM glycine (15 s, indicated by the arrow) followed 15 min later by a 5 min exposure to 100 μM glutamate/1 μM glycine. Experimental manipulations were performed during the 2 min immediately following the second glutamate exposure. (a) In this representative control Ca^{2+} trace, the neurone was perfused with HBSS (20 ml min^{-1}) for the duration of the experiment. After the second glutamate exposure, methanol (0.05%, vehicle control) in Ca^{2+} free HBSS was applied during the 2 min after glutamate removal followed by drug-free Ca^{2+} free HBSS for the remainder of the experiment. (b–d) Representative traces recorded as in (a), except that 25 μM CGP-37157 (b), 20 μM trifluoperazine (c) or 100 μM dibucaine (d) was included during the 2 min following glutamate exposure. Data are representative of 4–8 neurones per condition, from at least 3 separate culture dates.

1996; White & Reynolds, 1996). These conclusions have been based on the ability of cyclosporin A to block glutamate-induced mitochondrial depolarization. However, cyclosporin A is less than ideal as a PTP inhibitor for several reasons, including its large size (11 amino acid polypeptide) with poor cell penetrability often requiring pre-incubation for full effect and its inhibition of calcineurin which complicates interpretation of its effects on PTP (Liu *et al*., 1991; Dawson *et al*., 1993; Ankarcrona *et al*., 1996). Here, we have investigated two drugs, trifluoperazine and dibucaine, which inhibit or delay the onset of glutamate-induced mitochondrial PTP in intact neurones. In isolated mitochondria, these two drugs inhibit PTP by a different mechanism than cyclosporin A (Broekemeier *et al*., 1989), possibly by inhibition of phospholipase A_2 (Broekemeier *et al*., 1985). Usually, both cyclosporin A and trifluoperazine or dibucaine are necessary in combination for long-lasting inhibition of PTP (Broekemeier & Pfeiffer, 1995), which is consistent with the short-lived effects of dibucaine and trifluoperazine seen in this study. In addition to effects of trifluoperazine on PTP in isolated mitochondria, inhibition of PTP in intact hepatocytes stimulated with t-butyl hydroperoxide (Nieminen *et al*., 1995) has been observed. The present study describes the first demonstration of the utility of these drugs in the inhibition of PTP induced in intact neurones by an endogenous neurotransmitter.

Another important consideration in the study of PTP in neurones is whether it plays an important role in the Ca^{2+} dynamics after glutamate removal. Activation of the PTP will inhibit Ca^{2+} uptake into the mitochondria due to the collapse of the membrane potential which drives uptake. Activation of the pore may also result in release of accumulated mitochondrial matrix Ca^{2+} (Savage & Reed, 1994). Our studies have demonstrated that the mitochondrial Na^+/Ca^{2+} exchanger plays a dominant role in regulating the rate of Ca^{2+} recovery after a glutamate-induced Ca^{2+} load by releasing the mitochondrial Ca^{2+} pool (White & Reynolds, 1997). We found that the effect of trifluoperazine on Ca^{2+} recovery was qualitatively similar to that of the specific mitochondrial Na^+/Ca^{2+} inhibitor, CGP-37157 (Figure 4b and d). It has been shown that trifluoperazine can inhibit mitochondrial Na^+/Ca^{2+} exchange in isolated mitochondria (Hayat & Crompton, 1985), and the effect of trifluoperazine on Ca^{2+} recovery is consistent with this suggestion. Our data represent the first demonstration of inhibition of mitochondrial Na^+/Ca^{2+} exchange by trifluoperazine in intact cells. Activation of the PTP could also alter the recovery of $[Ca^{2+}]_i$ following glutamate stimulation, by allowing the rapid release of the mitochondrial Ca^{2+} pool (Savage & Reed, 1994). However, dibucaine did not qualitatively change the characteristic of Ca^{2+} recovery. This suggests that dibucaine does not inhibit mitochondrial Na^+/Ca^{2+} exchange. Moreover, it seems unlikely that activation of PTP by glutamate is contributing substantially to the mechanism by which the neurone recovers from a glutamate-imposed Ca^{2+} load. Additionally, these results re-emphasize the importance of Ca^{2+} efflux from mitochondria via the mitochondrial Na^+/Ca^{2+} exchanger as the predominant mechanism that shapes the Ca^{2+} recovery.

Interestingly, both trifluoperazine and dibucaine caused mitochondrial membrane hyperpolarization in the absence of glutamate. The mechanism underlying this phenomenon is not clear. It is possible that there is a constitutive, but low level of activation of the PTP in resting neurones. By blocking this activity, mitochondrial hyperpolarization may result. It also seems likely that the inhibition of mitochondrial Na^+/Ca^{2+} exchange contributes to the effects of trifluoperazine on the mitochondrial membrane potential, because similar effects have been observed with CGP-37157 (White & Reynolds, 1996). This is likely to be mediated by inhibition of mitochondrial Ca^{2+} cycling and the resulting elevation of matrix Ca^{2+}, which can then regulate several aspects of glycolysis and electron transport (Gunter *et al*., 1994). However, dibucaine apparently did not alter mitochondrial Na^+/Ca^{2+} exchange. Unless inhibition of a low, constitutive level of PTP activation accounts for this effect, dibucaine must therefore regulate mitochondrial membrane potential by a different, as yet undefined, mechanism.

Trifluoperazine and dibucaine affect other cellular processes in addition to their effects on PTP. Specifically, trifluoperazine is an inhibitor of calmodulin (Levin & Weiss, 1979) and may exert more general inhibitory effects by competing for Ca^{2+} binding to specific Ca^{2+} recognition sites in proteins. Dibucaine is an inhibitor of voltage-sensitive Na^+ channels and normally considered to be a local anaesthetic (Ritchie & Greene, 1990). The fact that both of these drugs have inhibition of phospholipase A_2 in common and that phospholipase A_2 is involved in the activation of PTP (Broekemeier *et al*., 1985) and, also, that they had essentially identical effects on glutamate-induced mitochondrial depolarization (i.e. PTP activation), strongly suggests that our results represent inhibition of PTP and not some other process. We confirmed that these drugs do not directly interact with NMDA receptors, as well (Figure 3), which is a property associated with a number of trifluoperazine-like drugs (Reynolds & Miller, 1988).

In conclusion, we have characterized the effects of trifluoperazine and dibucaine on glutamate-induced mitochondrial depolarization and recovery from glutamate-induced Ca^{2+} loads. Given that activation of the PTP is a potential candidate for mediating cell death, these drugs may be useful tools for the study of the role of the mitochondrial PTP in neuronal responses to glutamate injury, provided that their specificity of action can be established.

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