Neuropharmacology and analgesia

Dimebon, an antihistamine drug, inhibits glutamate release in rat cerebrocortical nerve terminals

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The excessive release of glutamate is a critical element in the neuropathology of acute and chronic brain disorders. The purpose of the present study was to investigate the effect and possible mechanism of dimebon, an antihistamine with a neuroprotective profile, on endogenous glutamate release in the nerve terminals (synaptosomes) of the rat cerebral cortex. Dimebon inhibited the release of glutamate that was evoked by exposing the synaptosomes to the K+ channel blocker 4-aminopyridine, and this effect was prevented by chelating extracellular Ca2+ ions, and the vesicular transporter inhibitor bafilomycin A1. Dimebon inhibited depolarization-evoked increase in cytosolic free Ca2+ concentration, and the dimebon-mediated inhibition of glutamate release was prevented by the Cav2.2 (N-type) and Cav2.1 (P/Q-type) channel blocker α-conotoxin MVIIIC. The inhibitory action of dimebon on glutamate release was not due to its decreasing synaptosomal excitability, because dimebon did not alter the resting synaptosomal membrane potential or 4-aminopyridine-mediated depolarization. Furthermore, the dimebon effect on 4-aminopyridine-evoked glutamate release was prevented by the protein kinase C inhibitor, and dimebon substantially reduced the 4-AP-induced phosphorylation of protein kinase C. However, the dimebon-mediated inhibition of glutamate release was unaffected by the N-methyl-D-aspartate receptor agonist or antagonist. These results suggest that dimebon inhibits glutamate release from rat cortical synaptosomes by suppressing presynaptic voltage-dependent Ca2+ entry and protein kinase C activity. This implies that the inhibition of glutamate release is an additional pharmacological activity of dimebon that may play a critical role in the apparent clinical efficacy of this compound.

1. Introduction

Dimebon is a non-selective antihistamine drug that has been suggested as a therapeutic application for treating neurodegenerative disorders, including Alzheimer’s disease and Huntington’s disease (Doody et al., 2008; Kieburtz et al., 2010; Sabbagh and Shill, 2010). Dimebon exhibits numerous benefits for the central nervous system, specifically, neuroprotective effects. For example, dimebon stabilizes neuronal Ca2+ signals and mitochondrial function (Wu et al., 2008; Zhang et al., 2010), attenuates glutamate-, methamphetamine- and β-amyloid-induced neurotoxicity (Bachurin et al., 2001; Geldenhuys et al., 2012; Lermontova et al., 2001; Perez et al., 2012; Wu et al., 2008), and protects against N-methyl-D-aspartate (NMDA)-induced seizures (Bachurin et al., 2001). Furthermore, dimebon has been reported to improve cognitive function and delay the progress of brain degeneration in experimental models and clinical trials (Bachurin et al., 2001; Lermontova et al., 2000; Steel et al., 2013; Vignisse et al., 2011; Wang et al., 2011; Webster et al., 2011). However, the precise mechanism of action underlying these neuroprotective effects is unclear.

In the brain, glutamate is a major excitatory neurotransmitter that plays an important role in many brain functions such as synaptic plasticity, learning, and memory (Fonnum, 1984). However, in addition to the physiological role of glutamate, excessive glutamate release and activation of the glutamate receptors induce an increase in intracellular Ca2+ levels, which subsequently triggers a cascade of cellular responses, including enhanced oxygen free radical production, disturbed mitochondrial function, and protease activation, which ultimately kill the neurons (Coyle and Puttfarcken, 1993; Schinder et al., 1996). This process has been
implicated as a pathophysiological factor in multiple neurological disorders, both acute, such as stroke and head trauma, and chronic, such as neurodegenerative disorders (Meldrum and Garthwaite, 1990; Meldrum, 2000). Modulation of glutamate neurotransmission, such as inhibition of glutamate release at nerve terminals, may provide a potential target for neuroprotective action. Indeed, some neuroprotective drugs (e.g., riluzole, memantine, and minocycline) have been revealed to decrease glutamate release in rat brain tissues (González et al., 2007; Lu et al., 2010; Wang et al., 2004).

Because dimebon has a neuroprotective profile, and the excessive release of glutamate is a critical element in the neuropathology of acute and chronic brain disorders, an assessment of the effects of dimebon on glutamate release is warranted. The purpose of the present study was to use isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to determine whether dimebon affects glutamate release. By using this model, we examined the effects of dimebon on the levels of released glutamate, the synaptosomal plasma membrane potential, and the activation of voltage dependent Ca²⁺ channels. In addition, because the role of protein kinase C in presynaptic modulation has been demonstrated (Coffey et al., 1993; Vaughan et al., 1998), we also evaluated whether this signaling pathway is involved in the regulation of glutamate release by dimebon. Finally, because dimebon is known to be an NMDA receptor antagonist (Grigorenko et al., 2003; Wu et al., 2008), we examined whether the regulation of glutamate release by dimebon is linked to antagonism at the NMDA receptor.

2. Material and methods

2.1. Chemicals

Dimebon, α-conotoxin MVIIIC, dantrolene, bisindolylmaleimide I (FG109203X), 2-[(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), N-methyl-α-aspartate (NMDA), D(-)-2-amino-5-phosphonopentanoic acid (α-AP5), and DL-threo-beta-benzyl-oxyaspartate (DL-TBOA) were obtained from Tocris Cookson (Bristol, UK). 3',3',3'-dipropylthiadicarbocyanine iodide ([DiSC₃(5)], and fura-2-acetoxyethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA, USA). The anti-phospho-protein kinase C (pan) rabbit polyclonal antibody directed protein kinase C-α, -β₁, -β₂, -δ, -ε, -η, and -i, phosphorylated at a carboxy-terminal residue homologous to Ser-660 of protein kinase C-β₁ was from Novus (Littleton, USA). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from BioRad (Milan, Italy). Ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Animals

Adult male Sprague-Dawley rats (150–200 g) were used in this study. All of the animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Fu Jen Institutional Animal Care and Utilization Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Synaptosomal preparation

Synaptosomes were prepared as described previously (Chiu et al., 2011; Nicholls et al., 1987). Briefly, the animals were killed by decapitation and the cerebral cortex were rapidly removed at 4 °C. The tissue was homogenized in a medium containing 320 mM sucrose, pH 7.4. The homogenate was centrifuged at 3000 g (5000 rpm in a JA 25.5 rotor; Beckman Coulter, Inc., USA) for 10 min at 4 °C, and the supernatant was centrifuged again at 14,500 g (11,000 rpm in a JA 25.5 rotor) for 12 min at 4 °C. The pellet was gently resuspended in 8 ml of 320 mM sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was placed into 3 ml Percoll discontinuous gradients containing 320 mM sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3%, 10% and 23% Percoll, pH 7.4. The gradients were centrifuged at 32,500 g (16,500 rpm in a JA 20.5 rotor) for 7 min at 4 °C. Synaptosomes sedimenting between the 10 and the 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 6H₂O, 1.2 mM Na₂HPO₄, 10 mM glucose, and 10 mM HEPES (pH 7.4). Protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets containing 0.5 mg of protein. Synaptosomal pellets were stored on ice and used within 4–6 h.

2.4. Glutamate release

Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that employed exogenous glutamate dehydrogenase (GDH) and NADP⁺ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically (Nicholls et al., 1987). Synaptosomal pellets were resuspended in HEPES buffer medium that contained 16 μM bovine serum albumin and incubated in a stirred and thermostatted cuvette maintained at 37 °C in a Perkin-Elmer LS-55 spectrophotometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). NADP⁺ (2 mM), GDH (50 units/ml) and CaCl₂ (1 mM) were added after 3 min. In experiments that investigated Ca²⁺-independent efflux of glutamate, EGTA (200 μM) was added in place of CaCl₂. Other additions before depolarization were made as described in the figure legends. After a further 10 min of incubation, 4-aminopyridine (1 mM), or KCl (15 mM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 nm and 460 nm, respectively) caused by NADPH being produced by oxidative deamination of released glutamate by GDH. Data were accumulated at 2 s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate was expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and depicted in bar graphs represent the levels of glutamate cumulatively released after 5 min of depolarization, and are expressed as nmol/mg/5 min. Estimation of the IC₅₀ was based on a one-site model [Inhibition = [Inhibition]MAX × [dimebon]/[IC₅₀ + [dimebon]]], and calculated using the nonlinear curve-fitting function provided in MicroCal Origin. Cumulative data were analyzed using Lotus 1-2-3.

2.5. Cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ)

The [Ca²⁺]ᵢ was measured using the Ca²⁺ indicator Fura-2. Synaptosomes (0.5 mg/ml) were resuspended in HEPES buffer medium containing 0.1 mM CaCl₂ and loaded with 5 μM Fura-2 for 30 min at 37 °C. The synaptosomes were washed with HEPES buffer medium by being centrifuged, resuspended in 2 ml of HEPES buffer medium containing bovine serum albumin, and placed in a Perkin-Elmer LS-55 spectrophotometer at 37 °C with stirring in the presence of 1.2 mM CaCl₂. The synaptosomes were incubated for 10 min in the presence of dimebon (10 μM) prior to being depolarized with 4-aminopyridine (1 mM). Fura-2-Ca fluorescence was determined at excitation wavelengths of 340 nm and 380 nm (emission wavelength, 505 nm), and data were accumulated at 2 s intervals. [Ca²⁺]ᵢ (nM)
was calculated by using calibration procedures (Sihra et al., 1992) and equations described previously (Gryniewicz et al., 1985). Cumulative data were analyzed using Lotus 1-2-3.

2.6. Synaptosomal plasma membrane potential

The plasma membrane potential was determined using a membrane-potential-sensitive dye, DiSC3(5) (Akerman et al., 1987). Synaptosomes were resuspended in HEPES buffer medium and incubated in a stirred and thermostated cuvette at 37 °C in a Perkin-Elmer LS-55 spectrophotometer. After 3 min of incubation, 5 μM DiSC3(5) were added and allowed to equilibrate before the addition of CaCl2 (1 mM) after 4 min of incubation. 4-aminopyridine was then added to depolarize the synaptosomes for 10 min, and DiSC3(5) fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm, respectively. Cumulative data were analyzed using Lotus 1-2-3 and expressed in fluorescence units.

2.7. Western blotting

Synaptosomes (0.5 mg protein/ml) from control and drug-treated groups were lysed in ice-cold Tris–HCl buffer solution, pH 7.5, that contained 20 mM Tris–HCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 1 μg/ml leupeptin. The lysates were sonicated for 10 s and then centrifuged at 13,000 g at 4 °C for 10 min. Equal amounts of samples were separated by electrophoresis on 7.5% SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline that contained 5% low-fat milk and incubated with appropriate primary antibodies (anti-phospho-protein kinase C (pan), 1:1000). After incubation with appropriate peroxidase-conjugated donkey anti-rabbit IgG secondary antibodies (1:3000), protein bands were detected by using the enhanced chemiluminescence method. An aliquot of samples was loaded and probed with anti-β-actin antibody for detection of β-actin as a loading control. Films were scanned using a scanner and the level of expression or phosphorylation was assessed by band density, which was quantified by densitometry.

2.8. Data analysis

Data were expressed as mean ± SEM. The data reported were analyzed by using the unpaired Student’s t test or by using two-way ANOVA accompanied by Tukey’s test for multiple comparisons. Analysis was completed via software SPSS (17.0; SPSS Inc., Chicago, IL). A value of P < 0.05 was considered to be significant.

3. Results

3.1. Dimebon inhibits 4-aminopyridine-evoked glutamate release from rat cerebrocortical synaptosomes; this effect is due to a decrease in vesicular exocytosis

To investigate the influence of dimebon on glutamate release, isolated nerve terminals were depolarized with the K+-channel blocker 4-aminopyridine. 4-aminopyridine destabilizes the membrane potential and is thought to cause repetitive spontaneous Na+ channel-dependent depolarization that closely approximates in vivo depolarization of the synaptic terminal that leads to the activation of voltage-dependent Ca2+ channels and neurotransmitter release (Tibbs et al., 1989). In synaptosomes incubated in the presence of 1 mM CaCl2, 4-aminopyridine (1 mM) evoked a glutamate release of 7.9 ± 0.1 nmol/mg/5 min. Application of dimebon (10 μM) reduced the amount of 4-aminopyridine-evoked glutamate release to 4.1 ± 0.2 nmol/mg/5 min [t(8) = 14.34, P = 0.000001] without altering the basal release of glutamate (Fig. 1A). The dimebon-induced inhibition of 4-aminopyridine-evoked glutamate release was concentration-dependent, and produced an IC50 value of approximately 6 μM, which was derived from a dose-response curve (Fig. 1B).

We next investigated whether the inhibition of 4-aminopyridine-evoked glutamate release by dimebon was mediated by an effect on exocytotic vesicular release, or on Ca2+-dependent release attributable to cytosolic efflux via reversal of the glutamate transporter (Nicholls et al., 1987). In Fig. 2A, the Ca2+-dependent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-aminopyridine in extracellular-Ca2+-free solution that contained 300 μM EGTA. Under these conditions, the release of glutamate evoked by 4-aminopyridine (1 mM) was 2.4 ± 0.3 nmol/mg/5 min. This Ca2+-dependent release evoked by 4-aminopyridine was, however, not affected by 10 μM dimebon (2.0 ± 0.4 nmol/mg/5 min) [t(6) = 0.95, P=0.38] (Fig. 2A). In Fig. 2B and C, we investigated the action of dimebon in the presence of DL-TBOA, a non-selective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, or bafloxymycin A1, which causes the depletion of glutamate in synaptic vesicles. Fig. 2B shows that 4-aminopyridine-evoked glutamate release was increased by 10 μM DL-TBOA (because of inhibition of reuptake of released glutamate) [F(1,15) = 112.4, P = 0.000]. In the presence of DL-TBOA, dimebon was able to produce

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Fig. 1. Dimebon inhibits 4-aminopyridine-evoked release of glutamate in rat cerebrocortical nerve terminals. (A) Glutamate release was evoked by the addition of 1 mM 4-aminopyridine in the absence (control) and in the presence of dimebon (10 μM), added 10 min prior to the addition of 4-aminopyridine. (B) Dose–response curve for dimebon inhibition of 4-aminopyridine-evoked glutamate release. Results are mean ± SEM of 5 independent experiments. ***P < 0.001 versus control group.
a 41.5 ± 3.8% inhibition on 4-aminopyridine-evoked glutamate release, which was similar to that observed for the 45.1 ± 2.5% inhibition produced by dimebon (10 μM) alone [F(1,15)=3.92, P=0.53; Fig. 2B and D]. In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced 4-aminopyridine-evoked glutamate release [F(1,15)=554.95, P=0.000, Fig. 2C]. In the presence of bafilomycin A1, however, dimebon (10 μM) only produced a 4.7 ± 2.3% decrease in the 4-aminopyridine-evoked glutamate release, which was less than that the inhibition produced by dimebon alone (45.1 ± 2.5%) [F(2,23)=12.28, P<0.05, Fig. 2C and D]. These results suggest that the inhibition of glutamate release by dimebon is mediated by a reduction in the Ca²⁺-dependent exocytotic component of glutamate release.

### 3.2. Dimebon reduces 4-aminopyridine-induced increase in intrasynaptosomal Ca²⁺ levels but does not change the synaptosomal membrane potential

Transmitter release can be modulated by regulating the plasma membrane potential, and consequently altering the calcium influx. To investigate the potential mechanisms underlying the dimebon-mediated inhibition of glutamate release, the effect of dimebon on intrasynaptosomal Ca²⁺ levels was determined by using the Ca²⁺ indicator Fura-2. In Fig. 3A, 4-aminopyridine (1 mM) caused a rise in cytosolic Ca²⁺ concentration ([Ca²⁺]c) from 145.4 ± 1.5 nM to a plateau level of 202.7 ± 4.5 nM (P<0.001). Applying dimebon (10 μM) did not affect basal Ca²⁺ levels, but caused an approximately 20% decrease in the 4-aminopyridine-evoked rise in [Ca²⁺]c (173.7 ± 5.8 nM; t(8)=53.5, P=0.001; Fig. 3A). The inhibition of the [Ca²⁺]c elevation by dimebon might be attributed either to a direct reduction in the amount of Ca²⁺ entering through voltage-dependent Ca²⁺ channels, or to secondary effects caused by, for example, the modulation of potassium channels and the consequently altered plasma membrane potential. To discern between these two possibilities, the effect of dimebon on the synaptosomal plasma membrane potential under resting conditions and on depolarization was examined using membrane potential-sensitive dye DiSC₃(5). Fig. 3B shows that 4-aminopyridine (1 mM) caused an increase in DiSC₃(5) fluorescence of 9.1 ± 0.5 fluorescence units/5 min. Preincubation of the synaptosomes with dimebon (10 μM) for 10 min before adding 4-aminopyridine did not alter the resting membrane potential, and produced no substantial change in the 4-aminopyridine-mediated increase in DiSC₃(5) fluorescence (9.2 ± 0.4 units/5 min; t(8)=−1.56, P=0.82). This suggests that the
observed inhibition of 4-AP-evoked glutamate release by dimebon is unlikely to be due to a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential or an attenuation of depolarization produced by 4-aminopyridine. Confirmation that the dimebon effect did not impinge on synaptosomal excitability was obtained with experiments using high external [K+]i-mediated depolarization. Elevated extracellular KCl depolarizes the plasma membrane by shifting the K\(^+\) equilibrium potential above the threshold potential for activation of voltage-dependent ion channels. While Na\(^+\) channels are inactivated under these conditions, voltage-dependent Ca\(^{2+}\) channels are activated nonetheless to mediate Ca\(^{2+}\) entry, which supports neurotransmitter release (Barrie et al., 1991). In Fig. 3B (inset), KCl (15 mM) evoked a glutamate release of 8.0 ± 0.4 nmol/mg/5 min, which was reduced to 5.1 ± 0.9 nmol/mg/5 min in the presence of 10 μM dimebon (F_0(8) = 3.1, P = 0.02).

3.3. A reduction of calcium influx through Cav\(_{2.2}\) (N-type) and Cav\(_{2.1}\) (P/Q-type) channels is involved in the action of dimebon on glutamate release

In the adult rat cerebrocortical nerve terminals, the release of glutamate evoked by depolarization is supported by Ca\(^{2+}\) influx through Cav\(_{2.2}\) (N-type) and Cav\(_{2.1}\) (P/Q-type) channels (Millan and Sanchez-Prieto, 2002; Vazquez and Sanchez-Prieto, 1997). To determine whether the decrease in Ca\(^{2+}\) channel activity was involved in the effect of dimebon on 4-AP-evoked glutamate release, we examined the effect of dimebon in the presence of ω-conotoxin MVIIIC, a wide spectrum blocker of Cav\(_{2.2}\) (N-type) and Cav\(_{2.1}\) (P/Q-type) Ca\(^{2+}\) channels. In Fig. 4A, 4-aminopyridine (1 mM)-evoked glutamate release was substantially reduced in the presence of ω-conotoxin MVIIIC (2 μM) [F(1,13) = 229.58, P = 0.000, 0.0000]. Although the 4-aminopyridine-evoked glutamate release was considerably reduced in the presence of dimebon (10 μM), this effect was prevented by the presence of ω-conotoxin MVIIIC (Fig. 4A). On average, the inhibitory effect of dimebon on 4-aminopyridine-evoked glutamate release was reduced to 5.1 ± 1.9% in the presence of ω-conotoxin MVIIIC, which was significantly different from the effect of dimebon alone (44.1 ± 3.4%) [F(3,22) = 19.18, P < 0.05, Fig. 4D].

In addition to the Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels, the release of glutamate evoked by depolarization was reported to be caused by a Ca\(^{2+}\) release from intracellular stores such as endoplasmic reticulum and mitochondria (Berridge, 1998). Thus, we tested the effect of dantrolene, an inhibitor of intracellular Ca\(^{2+}\) release from endoplasmic reticulum, and 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), a membrane-permeant blocker of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange, on the dimebon-mediated inhibition of glutamate release. In Fig. 4B and C, 4-aminopyridine (1 mM)-evoked glutamate release was reduced by dantrolene (100 μM) [F(1,14) = 6.53, P = 0.002] or CGP37157 (100 μM) [F(1,12) = 11.91, P = 0.005]. In the presence of either dantrolene or CGP37157, dimebon (10 μM) still effectively inhibited 4-aminopyridine-evoked glutamate release by 39.5 ± 3.3% and 44.7 ± 6.3%, respectively, which was not significantly different from the inhibition of dimebon alone (44.1 ± 3.4%; P > 0.05; Fig. 4D). These results suggest that decrease in the Ca\(^{2+}\) release from intracellular stores appears not to mediate the inhibitory effect of dimebon on glutamate release.

3.4. Involvement of protein kinase C in the dimebon-mediated inhibition of glutamate release

Since the protein kinase C signaling cascade is known to be present at the presynaptic level and has a crucial role in neurotransmitter exocytosis (Coffey et al., 1993; Hung et al., 1998; Vaughan et al., 1998; Wang and Sihra, 2004), we investigated whether the cascade participated in dimebon-mediated inhibition of glutamate release. To this end, we studied the effect of dimebon following inhibition of protein kinase C. Consistent with previous studies, the protein kinase C inhibitor GF109203 (10 μM) reduced 4-aminopyridine (1 mM)-evoked glutamate release [F(1,12) = 43.59, P = 0.0000, Fig. 5A]. In the presence of GF109203 × , dimebon only reduced glutamate release by 1.7 ± 5.9%, indicating significant reduction compared with that obtained when dimebon was applied alone (45.1 ± 2.9%) [F(2,21) = 32.05, P < 0.05] (Fig. 5A and C). In contrast to the effect of protein kinase C inhibitor, the mitogen-activated protein kinase inhibitor PD98059 (50 μM) reduced 4-aminopyridine (1 mM)-evoked glutamate release [F(1,10) = 24.81, P = 0.001], but it failed to influence the ability of dimebon to inhibit 4-aminopyridine-evoked release of glutamate (Fig. 5B). In the presence of PD98059, dimebon was still able to reduce the 4-aminopyridine-evoked glutamate release by 45.9 ± 3.3%, which was not significantly different from the inhibition of dimebon alone (45.1 ± 2.9%; P > 0.05; Fig. 5C). To confirm that the protein kinase C signaling pathway was suppressed by dimebon during its inhibition of 4-aminopyridine-evoked glutamate release, we determined the effect of dimebon on the phosphorylation of protein kinase C in cerebrocortical synaptosomes. Fig. 5D shows that depolarization of synaptosomes with 4-aminopyridine (1 mM)
markedly increased the phosphorylation of protein kinase C (120.5 ± 3.2%; t(4) = 2.1, P = 0.002). When synaptosomes were pretreated with dimebon (10 μM) for 10 min before depolarization with 1 mM 4-aminopyridine, 4-aminopyridine-enhanced phosphorylation of protein kinase C was markedly decreased to 102.7 ± 2.4% [F(2,6) = 23.29, P = 0.001; Fig. 5D].

3.5. Dimebon-inhibited glutamate release is unlikely to involve interaction with NMDA receptors

Previous reports suggested that dimebon may act as an inhibitor of NMDA receptor (Grigorev et al., 2003; Wu et al., 2008). NMDA receptor activation has been shown to regulate glutamate release from nerve terminals (Breukel et al., 1998; Luccini et al., 2007; Sequeira et al., 2001). Thus, the final experiment was to examine whether NMDA receptor was involved in the observed dimebon-mediated inhibition of glutamate release. In Fig. 6A, the effect of dimebon on 4-aminopyridine-evoked glutamate release in the absence or presence of NMDA (a agonist of NMDA receptor) was compared. 4-aminopyridine-evoked glutamate release was increased by NMDA (100 μM) [F(1,16) = 25.92, P = 0.000]. In the presence of NMDA, dimebon was still able to reduce the 4-aminopyridine-evoked glutamate release by 46.3 ± 3.2%, which was not significantly different from the inhibition of dimebon alone (40.8 ± 6.6%) [F(2,24) = 2.01, P = 0.16, Fig. 6C and D]. Next, the NMDA receptor antagonist d-AP5 (100 μM) had no effect on either 4-aminopyridine-evoked glutamate release or inhibition thereof by dimebon (Fig. 6B). On average, dimebon resulted in a 36.8 ± 3.6% inhibition on 4-aminopyridine-evoked glutamate release after treatment with d-AP5, which was similar with the inhibition produced by dimebon alone (40.8 ± 6.6%) [F(2,24) = 2.01, P = 0.16, Fig. 6C).

4. Discussion

The release of glutamate from a presynaptic site is a possible target for the drug modulation of excitability and synaptic transmission in central neurons (Wu and Saggau, 1997). Therefore, the purpose of this study was to investigate the relationship between the antihistamine drug dimebon and the presynaptic modulation of glutamate release, and to determine the underlying molecular mechanisms. By preparing nerve terminals (synaptosomes) from rat cerebral cortex, this study is the first to report that dimebon preferentially inhibits depolarization-evoked glutamate release.
However, dimebon did not affect the basal release of glutamate from the nerve terminals, suggesting that dimebon might reduce the release of glutamate when it is triggered by neuronal activation. The synaptosome was applied because it is capable of accumulating, storing, and releasing neurotransmitters, and is devoid of functional glial and nerve cell body elements that might cause the findings to be misinterpreted because of modulatory loci at the non-neuronal, postsynaptic, or network levels (Nicholls and Sihra, 1986). Therefore, the synaptosome provides a useful model for directly studying the specific presynaptic regulation of neurotransmitter release.

4.1. Mechanism of dimebon-mediated inhibition of glutamate release

In nerve terminals, the inhibition of Na\(^+\) channels or activation of K\(^+\) channels stabilizes membrane excitability and, consequently, causes a reduction in the levels of Ca\(^{2+}\) entry and neurotransmitter release (Li et al., 1993; Fong et al., 1999; Rehm and Tempel, 1991). The observed inhibitory effect of dimebon on evoked glutamate release could occur through a reduction of nerve terminal excitability, but this is unlikely because of the following 3 observations: First, 4-aminopyridine- versus KCl-evoked glutamate release were significantly inhibited by dimebon. Although 4-aminopyridine-evoked glutamate release involves the activation of Na\(^+\) and Ca\(^{2+}\) channels, 15 mM external KCl-evoked glutamate release involves only Ca\(^{2+}\) channels (Barrie et al., 1991; Nicholls, 1998), and this indicates that Na\(^+\) channels are not involved in the inhibitory effect of dimebon on glutamate release; Second, no substantial dimebon effect on synaptosomal plasma membrane potential was observed, which indicated a lack of effect on the K\(^+\) conductance; Third, dimebon did not affect the 4-aminopyridine-evoked Ca\(^{2+}\)-independent glutamate release, a component of glutamate release that is dependent only on membrane potential (Nicholls et al., 1987). This indicates that dimebon does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. This suggestion was supported by the observation that the inhibitory effect of dimebon on 4-aminopyridine-evoked glutamate release was prevented by the vesicular transporter inhibitor.
bafilomycin A1, but not by the glutamate transporter inhibitor DL-TBOA. Thus, these data suggest that the dimebon-mediated inhibition of 4-aminopyridine-evoked glutamate release is mediated by a decrease in the Ca^{2+}-dependent exocytotic component of glutamate release. Moreover, this phenomenon is not the result of a reduction in synaptosomal excitability caused by ion channel (e.g., the Na^{+} or K^{+} channels) modulation.

Therefore, if the effect is not caused by the modulation of synaptosomal excitability, then dimebon possibly inhibits evoked glutamate release by decreasing the levels of Ca^{2+} entry through the Ca_{2,2} (N-type) and Ca_{2,1} (P/Q-type) Ca^{2+} channels that are coupled to glutamate exocytosis in the nerve terminals (Millan and Sanchez-Prieto, 2002; Vazquez and Sanchez-Prieto, 1997). This hypothesis is plausible because the present study demonstrated that dimebon decreased the 4-aminopyridine-evoked increase in [Ca^{2+}]_{c}.

Furthermore, the inhibitory effect of dimebon on 4-aminopyridine-evoked glutamate release was prevented by ω-conotoxin MVIIIC, a wide spectrum blocker of the Ca_{2,2} (N-type) and Ca_{2,1} (P/Q-type) Ca^{2+} channels. However, neither dantrolene, an inhibitor of intracellular Ca^{2+} release from the endoplasmic reticulum ryanodine receptors, nor CGP37157, a mitochondrial Na^{+}/Ca^{2+} exchange blocker, affected the inhibitory effect of dimebon on glutamate release. Thus, the participation of reduced release of stored Ca^{2+} from the endoplasmic reticulum and mitochondria during the dimebon-mediated inhibition of glutamate release could be excluded. Based on these results, we suggest that dimebon inhibits evoked glutamate release by reducing presynaptic Ca_{2,2} (N-type) and Ca_{2,1} (P/Q-type) Ca^{2+} channel activity. Our finding is consistent with previous studies, which have shown that dimebon inhibits glutamate-induced Ca^{2+} increase and high voltage-activated Ca^{2+} channels in cultured neurons (Lermontova et al., 2001; Wu et al., 2008).

A role for the protein kinase C pathway in the dimebon-mediated inhibition of glutamate release is suggested in this study, based on the following results: (1) the inhibitory effect of dimebon on 4-aminopyridine-evoked glutamate release was substantially prevented by the protein kinase C inhibitor GF109203, but not by the mitogen-activated protein kinase inhibitor PD98059; and (2) dimebon significantly decreased 4-aminopyridine-induced phosphorylation of protein kinase C. Protein kinase C is one of the important intracellular signaling system at the presynaptic level, and plays a crucial role in neurotransmitter exocytosis (Coffey et al., 1993; Millan et al., 2003; Pittaluga et al., 2005; Sanchez-Prieto et al., 1996; Wang and Sihra, 2004). It has been shown that depolarization-stimulated Ca^{2+} entry enhances protein kinase C-dependent phosphorylation and glutamate release (Coffey et al., 1994). Thus, we suggest that the inhibitory effect of dimebon on Ca^{2+} entry observed here may decrease protein kinase C activity and, in turn, glutamate release. In addition, protein kinase C is capable of phosphorylating numerous synaptic proteins involved in the synaptic vesicle trafficking/recruitment and exocytosis, including MARCKS, SNAP-25, synapsin I, and munc18/Sec1. The phosphorylation of these synaptic proteins promotes the dissociation of synaptic vesicles from the actin cytoskeleton. This in turn makes more vesicles available for recycling.
available at the active zone for neurotransmitter exocytosis, resulting in an increased glutamate release (Craig et al., 2003; Jarvis and Zamponi, 2001; Vaughan et al., 1998). Therefore, it is possible that dimebon inhibits glutamate release by decreasing protein kinase C-dependent phosphorylation of synaptic proteins and the availability of synaptic vesicles.

It has been suggested that dimebon may act by blocking NMDA receptors (Grigorev et al., 2003; Wu et al., 2008). NMDA receptors are expressed in the brain and localize both pre- and post-synaptically (Engelman and MacDermott, 2004; Pinheiro and Mulle, 2008). At the presynaptic level, activation of NMDA receptors modulates Ca2+ influx and glutamate release (Breukel et al., 1998; Luccini et al., 2007; Sequeira et al., 2001). Therefore, the question arises as to whether NMDA receptors play any role in the dimebon-mediated inhibition of glutamate release. In the present study, the NMDA receptor agonist dimebon increased 4-AP-evoked glutamate release. This finding is consistent with previous studies (Breukel et al., 1998; Luccini et al., 2007). However, the inhibitory effect of dimebon on 4-aminopyridine-evoked glutamate release does not seem to involve NMDA receptors because it was obtained in the presence of the NMDA receptor agonist and antagonist. Our finding, although contrasting with previous studies (Grigorev et al., 2003; Wu et al., 2008), is consistent with a report by Giorgetti et al. (2010), showed that dimebon fails to block NMDA-induced Ca2+ influx in primary neuronal cells. The discrepancy is not clear, but may be related to the different experimental models applied and the expression of NMDA receptor subunits. In addition, the data in this study show that the NMDA receptor antagonist d-AP5 failed to affect the 4-aminopyridine-evoked glutamate release. Although the reason for this is unclear, probably because we depolarized synaptosomes with 1 mM 4-aminopyridine that closely simulates physiological stimulation (Silhara et al., 1992). In fact, under physiological conditions, glutamate acts on both the NMDA and non-NMDA receptors. However, the NMDA receptors are blocked by Mg2+, they only activated by the sustained depolarization. Consistent with this, our study and Breukel et al. (1998) had found that NMDA, at relatively high concentrations (100 μM), increases the release of glutamate evoked by 4-aminopyridine.

Although the data presented here suggest that antagonism at the NMDA receptor is unlikely mechanism for the inhibition of glutamate release produced by dimebon, the possible involvement of other neurotransmitter receptors should be considered. Serotonin, dopamine, and norepinephrine receptors, for example, are reported to be inhibited by dimebon at a 10 μM concentration (Grigorev et al., 2003; Schaffhauser et al., 2009; Wu et al., 2008). Consistent with these reports, the concentration of dimebon used to inhibit glutamate release in our study was 10 μM. In this study, dimebon-mediated inhibition of glutamate release is dose dependent, maximal at 30 μM, with an IC50 of 6 μM. In addition, numerous pharmacological effects of dimebon are also observed in a higher concentration range (i.e., 50–100 μM), including inhibition of acetylcholinesterase, blockade of NMDA receptors and voltage-gated Ca2+ channels, stabilization of mitochondrial function, as well as attenuating glutamate- or amyloid-induced neuronal death (Bachurin et al., 2001, 2003; Grigorev et al., 2003; Lermontova et al., 2001; Perez et al., 2012; Wu et al., 2008). These effects are likely to be associated with the neuroprotective activity of dimebon (Bachurin et al., 2001; Grigorev et al., 2003; Lermontova et al., 2001), although the exact mechanism for this benefit remains to be explored. In this work, the ability of dimebon to decrease glutamate release from nerve terminals may explain, in part, its neuroprotective mechanism. This is because the excessive release of glutamate is a critical factor in the neuropathology of acute and chronic brain disorders (Meldrum, 2000). Hence, it may be reasonable to assume that dimebon may have the potential to protect brain against glutamate-induced neurotoxicity. Future studies are needed to make a direct link between the inhibitory effect of dimebon on glutamate release and the compound’s neuroprotective effect against excitotoxic insults.

4.2. Conclusion

The results of the current study indicate that dimebon inhibits glutamate release from rat cerebrocortical synaptosomes by suppressing presynaptic voltage-dependent Ca2+ channels and protein kinase C activity. This implies that the inhibition of glutamate release is an additional pharmacological activity of dimebon that may play a critical role in the apparent clinical efficacy of this compound.

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References


