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Carbamazepine inhibits L-type Ca^{2+} channels in cultured rat hippocampal neurons stimulated with glutamate receptor agonists

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Abstract

In order to better understand the mechanism(s) of action of carbamazepine (CBZ), we studied its effects on the increase in $[Ca^{2+}]_i$ and $[Na^+]_i$ stimulated by glutamate ionotropic receptor agonists, in cultured rat hippocampal neurons, as followed by indo-1 or SBFI fluorescence, respectively. CBZ inhibited the increase in $[Ca^{2+}]_i$ stimulated either by glutamate, kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), or *N*-methyl-D-aspartate (NMDA), in a concentration-dependent manner. In order to discriminate the effects of CBZ on the activation of glutamate receptors from possible effects on Ca^{2+} channels, we determined the inhibitory effects of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ changes in the absence or in the presence of CBZ. The presence of 1 μM nitrendipine, 0.5 μM ω-conotoxin GVIA (ω-CgTx GVIA), or of both blockers, inhibited the kainate-stimulated increase in $[Ca^{2+}]_i$ by 51.6, 32.9 or 68.7%, respectively. In the presence of both 100 μM CBZ and nitrendipine, the inhibition was similar (54.1%) to that obtained with nitrendipine alone, but in the presence of both CBZ and ω-CgTx GVIA, the inhibition was greater (54%) than that caused by ω-CgTx GVIA alone. However, CBZ did not inhibit the increase in $[Na^+]_i$ stimulated by the glutamate receptor agonists, but inhibited the increase in $[Na^+]_i$ due to veratridine. Tetrodotoxin, or MK-801, did not inhibit the influx of Na^+ stimulated by kainate, indicating that Na^+ influx occurs mainly through the glutamate ionotropic non-NMDA receptors. Moreover, LY 303070, a specific AMPA receptor antagonist, inhibited the $[Na^+]_i$ response to kainate or AMPA by about 70 or 80%, respectively, suggesting that AMPA receptors are mainly involved. Taken together, the results suggest that CBZ inhibits L-type Ca^{2+} channels and Na^+ channels, but does not inhibit activation of glutamate ionotropic receptors.

Keywords: Carbamazepine; Glutamate ionotropic receptors; Kainate; Voltage-sensitive Ca^{2+} channels; Voltage-sensitive Na^+ channels; $[Ca^{2+}]_i$; $[Na^+]_i$

1. Introduction

Carbamazepine (CBZ) is an antiepileptic drug (AED) that has been extensively used in neurology in the treatment of epilepsies and trigeminal neuralgia, and in psychiatry for the prophylatic treatment of affective and schizoaffective psychosis. CBZ limits sustained high-frequency repetitive-firing of sodium-dependent action potentials of central neurons in cell culture at therapeutic concentrations (MacDonald and Kelly, 1993; Morimoto et al., 1997). This effect is commonly ascribed to a voltage-dependent inhibitory effect on voltage-gated Na^+ channels (Kapetanovic et al., 1995; Kuo et al., 1997; Rush and Elliott, 1997). However, the mechanisms underlying the effects of this AED have not been completely identified, and are still a matter of debate. It has been shown that CBZ can inhibit Ca^{2+} channels (Elliott, 1990; Schirrmacher et al., 1993; Schumacher et al., 1998), enhance 4-aminopyridine-sensitive potassium currents in cortical neurons (Olpe et al., 1991), acts as an antagonist of adenosine A_1 receptors (Biber et al., 1996), attenuates cAMP production (Chen et al., 1996), induces the release of serotonin (Dailey et

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al., 1997a,b), and inhibits the release of glutamate (Waldmeier et al., 1996; Okada et al., 1998). Preischemic treatment with CBZ also tends to reduce the cerebral damage and neurological deficit (Minato et al., 1997).

The demonstration by Croucher et al. (1982) that *N*-methyl-D-aspartate (NMDA) receptor antagonists had powerful anticonvulsant activity in a variety of animal seizure models, firmly established the concept that NMDA receptors play an important role in epileptic phenomena. More recently, the availability of selective non-NMDA receptor antagonists has made it possible to demonstrate that non-NMDA receptors also participate in seizure generation and are potential targets for antiepileptic drugs (Turski et al., 1987; Löscher, 1998). Various lines of evidence indicate that CBZ inhibits NMDA-induced responses (Lampe and Bigalpe, 1990; Gean et al., 1993; Hough et al., 1996). However, very little is known about the effect of CBZ on non-NMDA receptors. Considering this, and that the hippocampus has the highest density of kainate-binding sites in the brain (Ben-Ari, 1985), in the present work we investigated the effect of CBZ on $[Ca^{2+}]_i$ and $[Na^+]_i$ signals stimulated by ionotropic glutamate receptor agonists in cultured rat hippocampal neurons, in order to better understand the mechanism(s) of action of CBZ. The results indicate that CBZ inhibits L-type Ca^{2+} channels, which are activated subsequent to membrane depolarization due to glutamate receptor activation, at least at concentrations above 30 μM, but does not affect glutamate ionotropic receptor activation. A preliminary account of this work has been presented (Ambrósio et al., 1998).

2. Methods

2.1. Preparation and culture of hippocampal neurons

To prepare the hippocampal neurons in culture, cells were dissociated from hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.5 mg/ml, 15 min, 37°C) and deoxyribonuclease I (0.20 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4{\cdot}2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, supplemented with 0.001% phenol red, 1 mM sodium pyruvate, 10 mM Hepes, pH 7.4). The cells were cultured in serum-free Neurobasal medium (GIBCO), supplemented with B27 supplement (GIBCO), glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml). This procedure produces a nearly pure neuronal population as glial growth is reduced (Brewer et al., 1993). Cultures were kept at 37°C in a humidified incubator in 5% CO_2/95% air, for 7–8 days, the time required for maturation of hippocampal neurons.

Cells were plated on poly-D-lysine-coated (0.1 mg/ml) glass coverslips at a density of $0.2x10^6$ cells/cm^2 for indo-1 and SBFI fluorescence.

2.2. $[Ca^{2+}]_i$ measurements

Measurements of $[Ca^{2+}]_i$ were carried out in populations of hippocampal neurons cultured on glass coverslips at a density of 0.2×10^6 cells/cm^2. The cells were incubated with 3 μM indo-1/AM in Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM Hepes–Na, pH 7.4) for 40 min at 37°C, and the cells were further incubated for 20 min in Krebs buffer to obtain a complete hydrolysis of indo-1/AM. The coverslips were rinsed with Krebs buffer and mounted, with a special holder (Perkin-Elmer L2250008), in a temperature-controled cuvette. The fluorescence was monitored with a Spex Fluoromax spectrofluorometer, with excitation at 335 nm and emission at 410 nm, using 5 nm slits. The $[Ca^{2+}]_i$ was calculated with the following equation (Gelfand et al., 1986):

$$[Ca^{2+}]_i = 250 \times (F - F_{min})/(F_{max} - F), \text{ where}$$

$$F_{min} = AF + 1/12(F_{max} - AF)$$

The value of F_{max} was calculated upon addition of 3 μM ionomicin and the autofluorescence (AF) was measured in the presence of 3 mM $MnSO_4$.

Hippocampal neurons were incubated with the drugs 10 min before stimulation with the ionotropic glutamate receptor agonists until the end of each experiment. The calibration was made 100 s after stimulation with the agonists, using ionomicin and $MnSO_4$.

2.3. SBFI fluorescence measurements

Measurements of SBFI fluorescence were carried out in populations of hippocampal neurons cultured on glass coverslips at a density of 0.2×10^6 cells/cm^2. The cells were incubated with 10 μM SBFI/AM and 0.2% Pluronic F-127, in Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM Hepes–Na, pH 7.4), for 150 min at 37°C, followed by 15 min in Krebs buffer without SBFI/AM. The coverslips were rinsed with Krebs buffer and mounted, with a special holder (Perkin-Elmer L2250008), in a temperature-controled cuvette. The fluorescence was monitored with a Spex Fluoromax spectrofluorometer, with excitation wavelengths of 340 and 380 nm (in the ratio mode) and emission at 505 nm, with excitation and emission slits of 5 and 10 nm, respectively. Hippocampal neurons were incubated with the drugs at least 5 min before stimulation with the ionotropic glutamate receptor agonists until the end of each experiment. The variation of the excitation fluorescence ratio represents the difference between the values obtained 200 s after stimulation and basal values.

2.4. Chemicals

Carbamazepine (CBZ) was obtained from BIAL (S. Mamede do Coronado, Portugal). Glutamate, DNase (DN-25) and tetrodotoxin (TTX) were purchased from Sigma Chemical, St. Louis, MO, USA. Kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) were purchased from TOCRIS, Ballwin, MO 63011, USA. Nitrendipine was a kind gift of Dr G. Terstappen, from Bayer A.G, Germany. ω-conotoxin GVIA (ω-CgTx GVIA) was obtained from Peninsula Laboratories, Belmont, CA, USA and ω-Agatoxin IVA (ω-Aga IVA) was from Peptide Institute, INC., Osaka, Japan. LY 303070 was a kind gift of Lilly Research Laboratories, Indianapolis, IN 46285, USA. MK-801 was a kind gift of Merck Sharp and Dohme, USA. The acetoxymethyl esters of indo-1 (indo-1/AM) and SBFI (SBFI/AM) were purchased from Molecular Probes, Eugene, OR, USA. Ionomycin was purchased from Calbiochem-Boehringer, San Diego, USA. Neurobasal medium, B27 supplement and trypsin (USP grade) were purchased from GIBCOBRL, Life Technologies, Paisley PA4 9RF, Scotland. All other reagents were from Sigma Chemical, St. Louis, MO, USA or from Merck-Schuchardt, Germany.

CBZ, nitrendipine, LY 303070, indo-1/AM, SBFI/AM and ionomycin stock solutions were prepared in DMSO.

2.5. Statistical analysis

The data are expressed as means ± S.E.M. Statistical significance was determined by using an analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's post-tests, or with Student's *t*-test, as indicated in the figure legends.

3. Results

3.1. Characterization of the $[Ca^{2+}]_i$ response stimulated by glutamate ionotropic receptor agonists in cultured hippocampal neurons

We determined the alterations in the $[Ca^{2+}]_i$ stimulated by glutamate ionotropic receptor agonists (glutamate, kainate, AMPA or NMDA) and found that the maximal change in $[Ca^{2+}]_i$ caused by the agonists was 232.3 nM ($n = 5$), 165.5 nM ($n = 7$), 166.5 nM ($n = 7$) and 74.2 nM ($n = 6$), for glutamate, kainate, AMPA and NMDA, respectively (Figs. 1 and 2). The $[Ca^{2+}]_i$

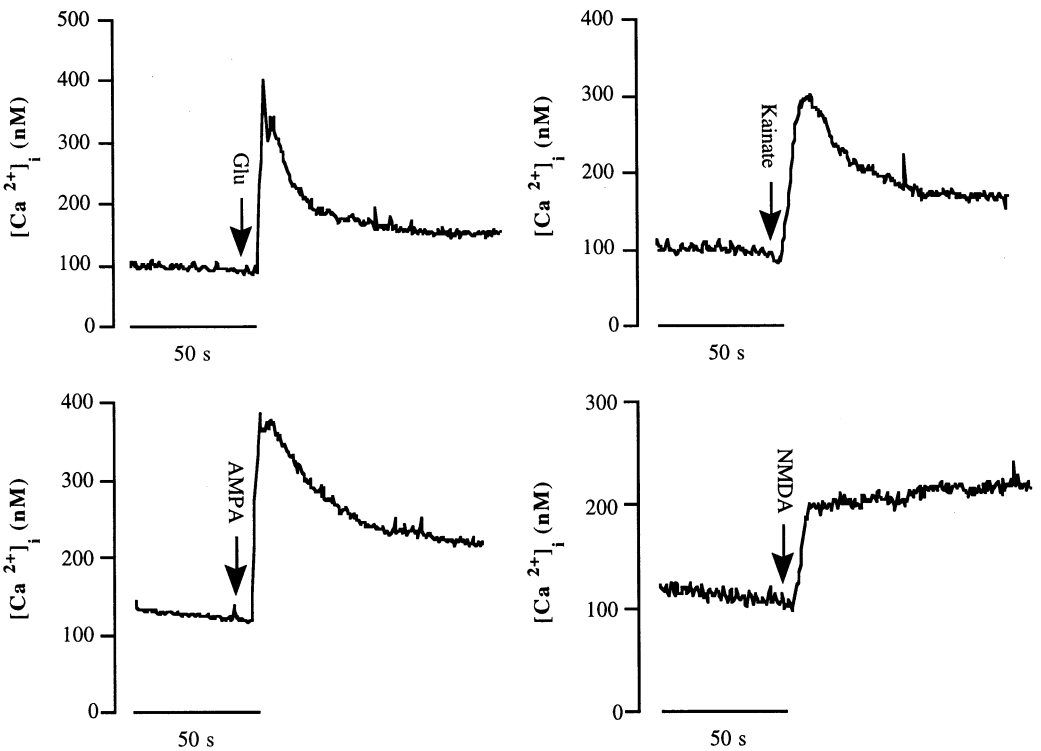

Fig. 1. Representative traces showing the increase in $[Ca^{2+}]_i$ stimulated by 100 μM of either glutamate (Glu), kainate, AMPA or NMDA, in cultured rat hippocampal neurons. The maximal change (average) in the $[Ca^{2+}]_i$, for the four agonists tested, was 232.3 nM ($n = 5$), 165.5 nM ($n = 7$), 166.5 nM ($n = 7$) and 74.2 nM ($n = 6$), respectively, and represents the difference between peak and basal $[Ca^{2+}]_i$ values. When the cells were stimulated with NMDA the experiments were performed in the absence of Mg^{2+}. The $[Ca^{2+}]_i$ was determined by using indo-1 fluorescence, and the equation developed by Gelfand et al. (1986), as indicated in Section 2.

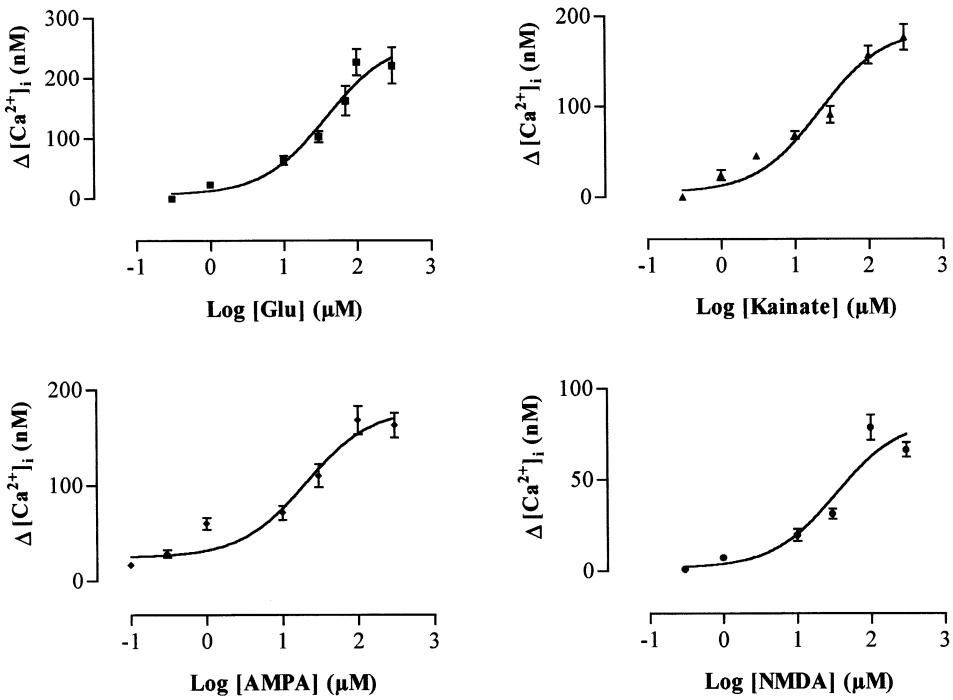

Fig. 2. Concentration-response curves for the effects of glutamate (Glu), kainate, AMPA or NMDA on peak $[Ca^{2+}]_i$ changes ($\Delta[Ca^{2+}]_i$). The EC_{97} values determined were 223.0 μM for Glu, 185.4 μM for KA, 201.4 μM for AMPA and 217.0 μM for NMDA. The EC_{50} for each agonist was 38.6 ± 1.6, 22.0 ± 1.4, 20.5 ± 1.5 and 33.7 ± 2.1 μM, respectively. When the cells were stimulated with NMDA, experiments were performed in the absence of Mg^{2+}. The curves indicate the best fits according to nonlinear regression analysis (sigmoidal dose-response curve). The results represent the mean ± S.E.M. of at least five independent experiments.

changes represent the difference between peak and basal $[Ca^{2+}]_i$ values. The experiments with NMDA were performed in the absence of Mg^{2+}. The calculated EC_{97} values were 223.0 μM for glutamate, 185.4 μM for kainate, 201.4 μM for AMPA and 217.0 μM for NMDA. The calculated EC_{50} values for each agonist were 38.6 ± 1.6, 22.0 ± 1.4, 20.5 ± 1.5 and 33.7 ± 2.1 μM, respectively.

3.2. Inhibition by CBZ of the $[Ca^{2+}]_i$ response stimulated by glutamate ionotropic receptor agonists

We determined the concentration-dependency effect of CBZ against glutamate, kainate, AMPA and NMDA utilized at the corresponding EC_{97} values determined above. CBZ inhibited the increase in the $[Ca^{2+}]_i$ induced by the glutamate receptor agonists in a dose-dependent manner (Fig. 3). The inhibitory effect of CBZ was greater in glutamate-stimulated neurons, as compared to the stimulation induced by the other agonists, probably because in glutamate-stimulated neurons the alterations in the $[Ca^{2+}]_i$ are greater. Thus, CBZ (500 μM), the highest concentration utilized, inhibited the increase in the $[Ca^{2+}]_i$ stimulated by 223.0 μM glutamate by $62.7 \pm 4.9\%$. In NMDA-stimulated neurons, only concentrations of CBZ above 100 μM caused an inhibition of the response, and the inhibition caused by 500 μM CBZ was $43.1 \pm 3.2\%$. The inhibition caused by 500 μM CBZ in hippocampal neurons stimulated either with kainate or AMPA was 52.2 ± 4.5 or $47.4 \pm 1.6\%$, respectively.

3.3. Characterization of the inhibitory effect caused by CBZ in kainate-stimulated neurons

Non-NMDA receptors have been implicated in epilepsy (Rogawski, 1995; Löscher, 1998). Kainate, a potent neurotoxin widely used in epilepsy studies, has a very high number of binding sites in the hippocampus (Ben-Ari, 1985). We found that the increase in the $[Ca^{2+}]_i$ due to stimulation with 100 μM kainate was inhibited by 60% in the presence of 15 μM LY 303070, a non-competitive AMPA receptor antagonist (not shown), suggesting that kainate-induced responses may be mediated by the activation of AMPA receptors. Considering that activation of ionotropic glutamate receptors could lead to the opening of voltage-sensitive Ca^{2+} channels (VSCCs), we investigated the contribution of different types of VSCCs to the increase in the $[Ca^{2+}]_i$, due to stimulation with kainate (185.4 μM, EC_{97}). The contribution of each Ca^{2+} channel type was determined in the presence of saturating concentrations of the three Ca^{2+} channel blockers utilized (Ambrósio et al., 1997). Thus, the increase of $[Ca^{2+}]_i$ stimulated by kainate was inhibited by $51.6 \pm 2.8\%$ in the presence of 1 μM nitrendipine (L-type Ca^{2+} channel blocker), and

by 32.9 ± 1.3% in the presence of ω-CgTx GVIA (0.5 μM), an N-type Ca^{2+} channel blocker (Fig. 4). The presence of both blockers caused an inhibition of 68.7 ± 2.9%. The P/Q-type Ca^{2+} channel blocker, ω-Aga IVA (100 nM) did not inhibit the increase of the $[Ca^{2+}]_i$ in these cells.

Since the presence of the Ca^{2+} channel blockers did not completely block the increase of the $[Ca^{2+}]_i$ caused by kainate, we checked other possible routes for the entry of Ca^{2+}. In order to investigate whether AMPA/kainate receptors, present in our preparation, were permeable to Ca^{2+}, we stimulated the hippocampal neurons with kainate in *N*-methyl-D-glucamine (NMG)-medium, where Na^+ is replaced by NMG, which does not enter into the cells, and we did not observe any increase in the $[Ca^{2+}]_i$ (not shown), indicating that in this preparation kainate or AMPA receptors are not permeable to Ca^{2+}.

Contrary to what we found for AMPA/kainate receptors, NMDA receptors in CNS neurons are highly permeable to Ca^{2+} (Ozawa, 1996). Considering also that activation of AMPA/kainate receptors could lead to membrane depolarization which, in turn, may unblock the NMDA receptor, we analysed the effect of MK-801 (10 μM), an antagonist of NMDA receptors, on the $[Ca^{2+}]_i$ increase caused by kainate. Fig. 5 shows that MK-801 did not have any effect (Mg^{2+} present in the medium), indicating that NMDA receptors do not contribute to the increase in the $[Ca^{2+}]_i$ under these conditions of stimulation.

Carbamazepine (100 μM) inhibited the $[Ca^{2+}]_i$ increase stimulated with kainate by 41.0 ± 4.2%. The inhibition caused by the presence of both CBZ and nitrendipine (54.1 ± 6.5%) was similar to that caused by nitrendipine alone (51.6 ± 2.8%). This result suggests that CBZ and nitrendipine are both acting on L-type Ca^{2+} channels. However, the inhibition produced in

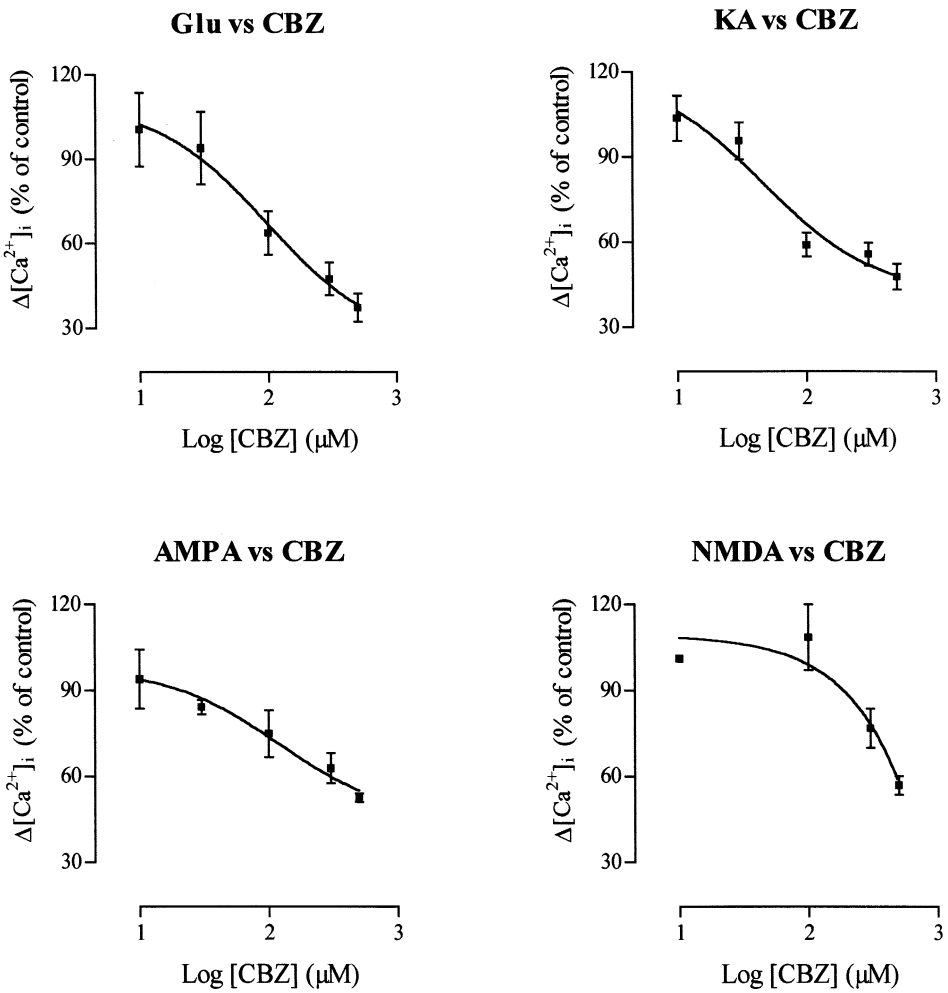

Fig. 3. Inhibition curves for the effect of CBZ on the $[Ca^{2+}]_i$ increase (% of control) due to glutamate receptor agonists. The agonist concentration chosen was the EC_{97} value in each case, as determined in Fig. 2: 223.0 μM glutamate (Glu), 185.4 μM kainate (KA), 201.4 μM AMPA or 217.0 μM NMDA. The curves represent the best fit according to nonlinear regression analysis (one site competition). The results represent the mean ± S.E.M. of at least four independent experiments.

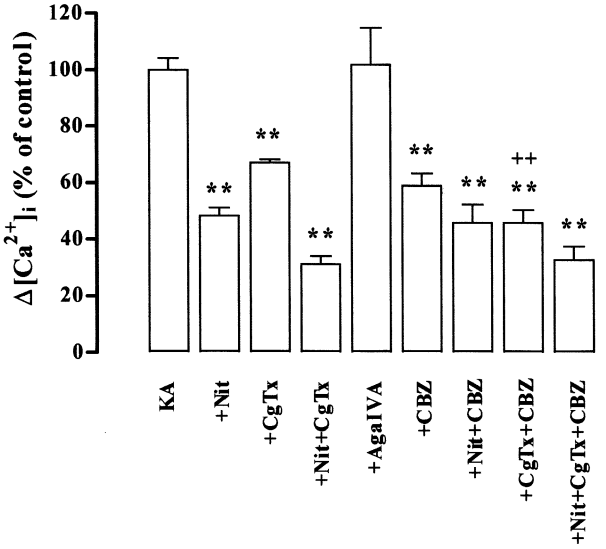

Fig. 4. Effect of the Ca^{2+} channel blockers nitrendipine (Nit, 1 μM), ω-CgTx GVIA (CgTx, 0.5 μM), ω-Agatoxin IVA (AgaIVA, 0.1 μM) and/or CBZ (100 μM) on the $[Ca^{2+}]_i$ increase stimulated by 185.4 μM kainate (EC_{97}; KA), in cultured rat hippocampal neurons. The cells were incubated with the Ca^{2+} channel blockers for 15 min before starting the $[Ca^{2+}]_i$ measurements. The results represent the mean ± S.E.M of at least five independent experiments. ** $P < 0.01$, Dunnett's post-test; $^{++}$ $P < 0.01$,Bonferronis's post-test; statistical significance when compared to 185.4 μM KA or 0.5 μM ω-CgTx GVIA, respectively.

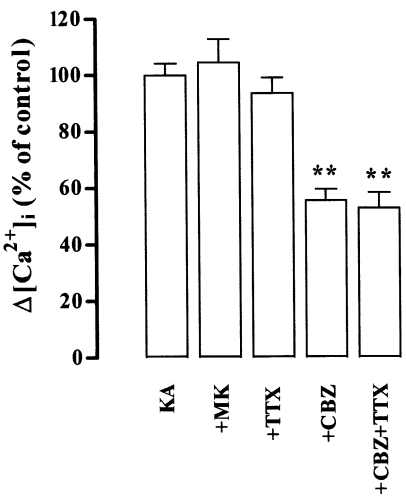

Fig. 5. Effect of MK-801 (MK, 10 μM), TTX (1 μM), CBZ (300 μM) and CBZ plus TTX on the $[Ca^{2+}]_i$ signal stimulated by 185.4 μM kainate (EC_{97}, KA), in cultured rat hippocampal neurons. The experiments were performed in the presence of Mg^{2+}. The results represent the mean ± S.E.M of at least five independent experiments. ** $P < 0.01$; statistical significance when compared to 185.4 μM KA (Dunnett's post-test).

the presence of both CBZ and ω-CgTx GVIA (54.0 ± 4.5%) was greater than that caused by ω-CgTx GVIA alone (32.9 ± 1.3%). This additive effect suggests that other types of Ca^{2+} channels different from the N-type, for example L-type Ca^{2+} channels, can be the target. Moreover, the inhibitory effect obtained in the presence of both CBZ, nitrendipine and ω-CgTx GVIA (67.1 ± 4.7%) was similar to that caused by a combination of both Ca^{2+} channel blockers (68.7 ± 2.9%), in the absence of CBZ. In these studies, we used 100 μM CBZ, since at this concentration CBZ significantly inhibited the response and is similar to the therapeutic range, i.e. plasma levels of 17–51 μM (Rogawski and Porter, 1990).

CBZ is known to inhibit voltage-sensitive Na^+ channels (VSSCs) (Kuo et al., 1997; Rush and Elliott, 1997). We therefore investigated the role of voltage-sensitive Na^+ channels on the increase in the $[Ca^{2+}]_i$, induced by kainate. Tetrodotoxin (1 μM), a blocker of VSSCs, did not cause any inhibition of the kainate effect, suggesting that these channels are not involved in the depolarization caused by kainate. Moreover, the inhibition caused by the presence of both CBZ and TTX is similar to that obtained with CBZ (Fig. 5). These results also indicate that the inhibitory effect of CBZ is not mediated through the inhibition of tetrodotoxin-sensitive Na^+ channels.

Thus, the results suggest that the main route for the entry of Ca^{2+} when the hippocampal neurons are stimulated with kainate are the VSCCs, namely L- and N-type Ca^{2+} channels, and the inhibitory effect caused by CBZ may be mediated by the inhibition of these channels, namely the L-type Ca^{2+} channels.

3.4. Characterization of the Na^+ influx stimulated by glutamate ionotropic receptor agonists

We observed that the increase in the SBFI excitation fluorescence ratio ($F_{340\ nm}/F_{380\ nm}$), as a measure of the $[Na^+]_i$ changes, is greater in hippocampal neurons stimulated with 100 μM glutamate as compared to 100 μM of either kainate, AMPA or NMDA (Fig. 6), and was, respectively (arbitrary units) 0.205 ± 0.011 ($n = 11$), 0.160 ± 0.007 ($n = 18$), 0.150 ± 0.005 ($n = 12$) or 0.075 ± 0.006 ($n = 11$). We then characterized the effects of CBZ on the $[Na^+]_i$ responses mediated by non-NMDA receptors in hippocampal neurons. Thus, when hippocampal neurons were stimulated either with 100 μM of kainate or 100 μM AMPA, the elicited $[Na^+]_i$ response was inhibited by 15 μM LY 303070 by 67.6 ± 2.9 or 79.1 ± 3.5%, respectively (Fig. 7). These results suggest that the Na^+ entry stimulated by kainate is essentially mediated by the activation of AMPA receptors. We also investigated the contribution of voltage-sensitive Na^+ channels and of NMDA receptors for the $[Na^+]_i$ signal stimulated by kainate, and observed that 1 μM TTX or 10 μM MK-801 (Mg^{2+} present in the medium) did not have any effect on the $[Na^+]_i$ signal (Fig. 7). These results indicate that NMDA receptors and voltage-sensitive Na^+ channels do not contribute significantly to the influx of Na^+,

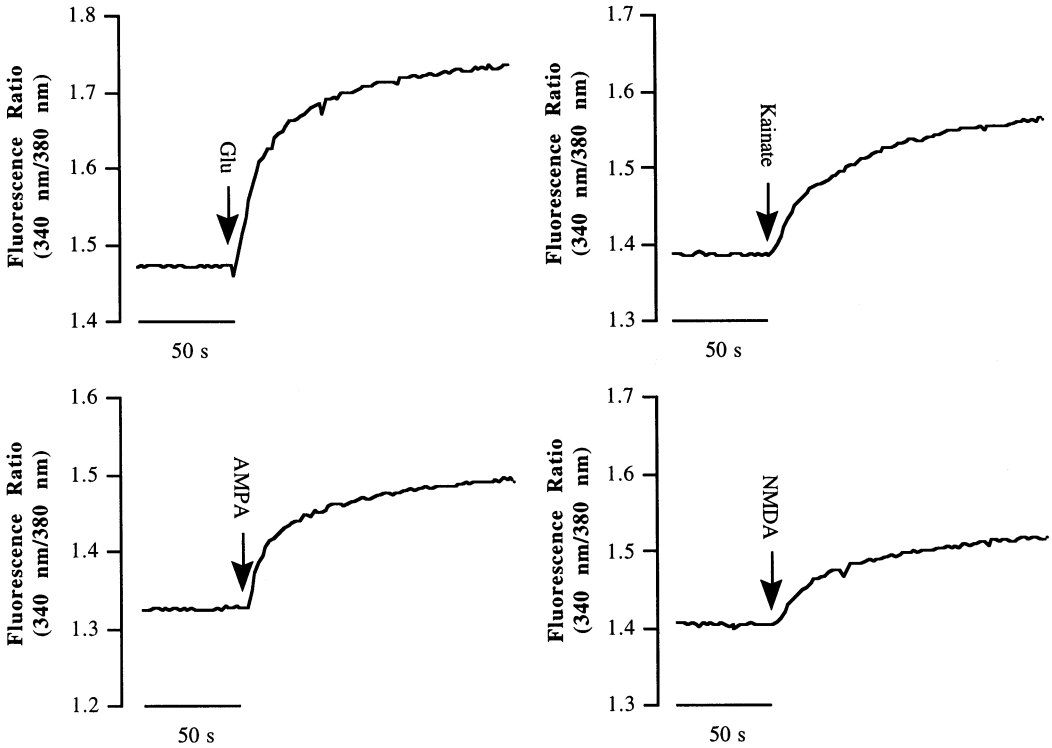

Fig. 6. Representative traces reflecting the increase in the $[Na^+]_i$ due to stimulation with 100 μM glutamate (Glu), kainate, AMPA or NMDA, in cultured rat hippocampal neurons, as followed by the excitation fluorescence ratio (340/380 nm) of SBFI. When cells were stimulated with NMDA the experiments were performed in the absence of Mg^{2+}. The experiments were performed as described in Section 2.

and the main route of Na^+ entry is the AMPA receptor channel.

3.5. Effect of carbamazepine on the $[Na^+]_i$ signal

CBZ (300 μM) did not inhibit the influx of Na^+ stimulated by 100 μM of either glutamate, kainate, AMPA or NMDA (Fig. 8), suggesting that CBZ, in our preparation, does not affect glutamate ionotropic receptors. We also analysed the effect of CBZ on the $[Na^+]_i$ signal stimulated by veratridine (25 μM), a Na^+ channel opener. CBZ inhibited the response by about 50% (not shown), confirming that CBZ also acts on Na^+ channels, and may interfere with these channels when they are specifically activated.

4. Discussion

CBZ blocks NMDA-induced currents in cultured spinal cord neurons (Lampe and Bigalpe, 1990), inhibits NMDA-induced depolarizations in cortical wedges prepared from DBA/2 mice (Lancaster and Davies, 1992), antagonizes convulsions produced by intracerebroventricular administration of NMDA (Sofia et al., 1994), and inhibits NMDA-evoked calcium influx in rat cerebellar granule cells (Hough et al., 1996). However, the inhibition caused by CBZ on NMDA-mediated responses appear to be independent of the NMDA and glycine recognition sites. Grant et al. (1992) showed that CBZ was inactive in displacing [^{3}H]dizocilpine at concentrations substantially higher

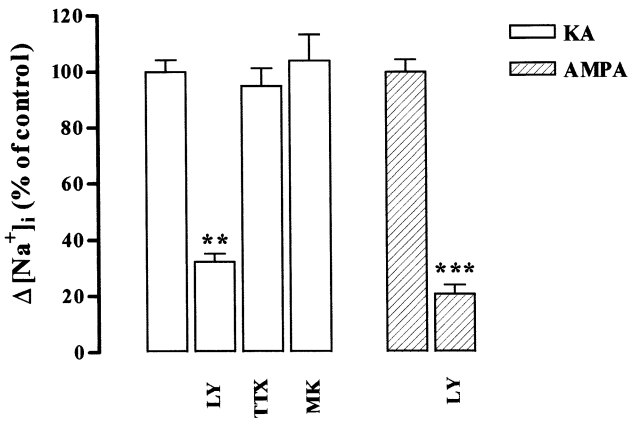

Fig. 7. Identification of the pathways of Na^+ entry in cultured rat hippocampal neurons, stimulated with kainate (KA, 100 μM) or AMPA (100 μM). Hippocampal neurons were stimulated with KA in the presence of either LY 303070 (LY, 15 μM), tetrodotoxin (TTX, 1 μM) or MK-801 (MK, 10 μM), and with AMPA (100 μM) in the presence of LY 303070 (15 μM). The results represent the mean ± S.E.M. of at least four independent experiments. ** $P < 0.01$, Dunnett's post-test; *** $P < 0.0001$, Sudent's t-test; statistical significance when compared to 100 μM KA or 100 μM AMPA, respectively.

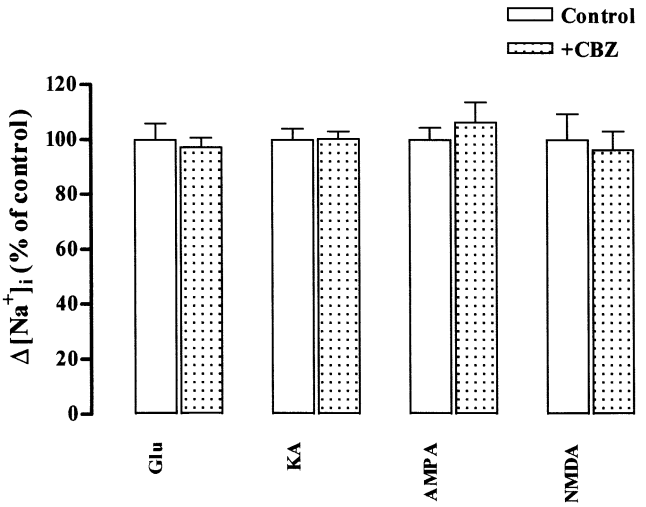

Fig. 8. Absence of effect of CBZ (300 μM) on the Na^+ influx stimulated by 100 μM of either glutamate (Glu), kainate (KA), AMPA or NMDA, in cultured rat hippocampal neurons. The experiments were performed as described in Section 2. The results represent the mean ± S.E.M. of at least four independent experiments.

than the therapeutic brain levels. Non-NMDA receptors are also involved in epileptiform activity (Rogawski, 1995; Löscher, 1998). Since little is known about the effect of CBZ on the responses mediated by non-NMDA receptors, we studied the effect of CBZ on the $[Ca^{2+}]_i$ and $[Na^+]_i$ signals stimulated by glutamate ionotropic receptor agonists in order to better understand the mechanism(s) of action of this AED.

4.1. Alterations in the $[Ca^{2+}]_i$ stimulated by glutamate ionotropic receptor agonists

First of all, we determined the alterations in $[Ca^{2+}]_i$ stimulated either by glutamate, kainate, AMPA or NMDA, and found that the maximal change in $[Ca^{2+}]_i$ was produced by glutamate, probably because it can activate both NMDA and non-NMDA receptors. The inhibitory effect of CBZ on glutamate-, kainate- or AMPA-induced $[Ca^{2+}]_i$ increases was similar, with some minor differences in the curve shape. CBZ caused a significant inhibition of the response only above 30 μM, but the effect was more pronounced in glutamate-stimulated neurons, at least with 300 and 500 μM CBZ, when compared to the effect on the responses evoked by the other agonists. This may be due to the higher $[Ca^{2+}]_i$ alterations observed in glutamate-stimulated neurons. We could not determine the IC_{50} values, since at concentrations above 500 μM the drug precipitated. The effect of CBZ on the NMDA-evoked $[Ca^{2+}]_i$ was different, since only concentrations above 100 μM CBZ produced an inhibitory effect, and the effect was smaller (Fig. 3). So, it seems that the extent of $[Ca^{2+}]_i$ alterations may be important for the effect of CBZ.

4.2. Effect of CBZ on kainate-mediated $[Ca^{2+}]_i$ responses

In the present work we extensively explored the effect of CBZ on the $[Ca^{2+}]_i$ changes due to kainate stimulation. Cai and McCaslin (1992) reported for the first time an interaction of tricyclic compounds with the function of glutamate receptors in concentrations used therapeutically, showing that CBZ prevented the elevation of the $[Ca^{2+}]_i$ induced by kainate. More recently, it was shown that CBZ also attenuated responses to AMPA in the rat cortical wedge (Phillips et al., 1997). We found that the responses caused by kainate stimulation are mainly due to AMPA receptor activation, and that L-type and N-type Ca^{2+} channels have a predominant role in the increase in the $[Ca^{2+}]_i$. The presence of both N- and L-type Ca^{2+} channel blockers inhibited the response by about 70%. Previous reports also show that L-type Ca^{2+} channels are activated upon stimulation with kainate (Murphy and Miller, 1989; Courtney et al., 1990). Concerning N-type Ca^{2+} channels there is no direct evidence showing an inhibitory effect produced by ω-CgTx GVIA in kainate-stimulated neurons. However, in our preparation it is clear that N-type Ca^{2+} channels are implicated in the alterations in the $[Ca^{2+}]_i$. Furthermore, it was previously shown that these channels play a small but significant role in neurotransmiter release induced by kainate (Keith et al., 1989). P-type Ca^{2+} channels, which are predominantly located in nerve terminals (Ambrósio et al., 1997) did not contribute to the increase in the $[Ca^{2+}]_i$ in the hippocampal cell cultures, probably because we are mainly looking at changes in neuronal cell bodies. R-type Ca^{2+} channels, which are essentially located in cell bodies (Yokoyama et al., 1995), could also be activated after a kainate stimulus. Unfortunatly, we could not check the contribution of R-channels, since Ni^{2+}, which is a specific blocker, at least at lower concentrations, interfered with the fluorescence recordings.

Since AMPA/kainate receptors may be permeable to Ca^{2+}, depending on the subunit composition, and NMDA receptors are highly permeable to Ca^{2+} (Lu et al., 1996), we checked whether Ca^{2+} could be entering through these receptors. Stimulation of hippocampal neurons with kainate in NMG-medium, or stimulation in the presence of MK-801 (Mg^{2+} present), showed that these receptors do not contribute, at least significantly, to the increase in the $[Ca^{2+}]_i$. It is important to note that endogenous glutamate can be released after stimulation with kainate and activate NMDA receptors (Malva et al., 1996). More recent evidence also suggests that ionotropic receptor-mediated Ca^{2+} signals in neurons might also involve release of Ca^{2+} from intracellular stores (Berridge, 1998). However, the present study clearly demonstrates that VSCCs have a predominant

role in these signals, namely L- and N-type Ca^{2+} channels (Fig. 4).

The inhibition obtained when both CBZ (100 μM) and nitrendipine were present was similar to the inhibition caused by the L-type Ca^{2+} channel blocker alone. These data indicate that CBZ, at least at higher concentrations, can affect L-type Ca^{2+} channels, and agree with other findings indicating that CBZ could have Ca^{2+} antagonistic properties (Elliott, 1990; Schirrmacher et al., 1993; Mark et al., 1995; Vonwegerer et al., 1997; Schumacher et al., 1998). Moreover, Schirrmacher et al. (1995) also suggested that CBZ reduces Ca^{2+} currents presumably by inhibiting L-type Ca^{2+} channels in rat sensory spinal ganglion cells.

The inhibition obtained in the presence of ω-Cgtx GVIA plus CBZ is greater (54%) as compared to the inhibition caused by the N-type Ca^{2+} channel blocker alone (33%), with the additional presence of CBZ not enhancing the inhibitory effect caused by both L- and N-type Ca^{2+} channel blockers. The additive effect of both CBZ and ω-CgTx GVIA further suggests that L-type Ca^{2+} channels can be the target for CBZ, but we cannot exclude the possibility that N-type Ca^{2+} channels can also be affected, since the effect is not completely additive. If they were affected however, the effect would be very small, because we observed that in the presence of nitrendipine, CBZ did not cause an additional inhibitory effect, as would be expected if N-type Ca^{2+} channels were also a target. However, Yoshimura et al. (1998) showed that CBZ inhibits high K^+-evoked secretion of catecholamines by interfering with N-type VSCCs. As mentioned above, the effects of CBZ were studied at a concentration of 100 μM, since the inhibitory effects were significant at this concentration, and because this concentration approximates to the plasma levels of 17–51 μM (Rogawski and Porter, 1990). Sayer et al. (1993) observed that CBZ produced a slight decrease in Ca^{2+} currents in cortical neurons, but only at concentrations of the order of 100 μM.

Since CBZ is known to inhibit voltage-sensitive Na^+ channels (Kuo et al., 1997; Rush and Elliott, 1997), the effect observed previously could be due to a reduction of the extent of depolarization. Thus, we analyzed the role of TTX-sensitive Na^+ channels on the increase of the $[Ca^{2+}]_i$ induced by kainate, and found that they are not involved in the depolarization caused by kainate, probably because they inactivate after the onset of depolarization. There are also TTX-insensitive and TTX-resistant Na^+ channels, but they are mainly found in preparations of the peripheral nervous system, and not from those of the CNS (Yoshida, 1994; Scholz et al., 1998). High K^+ directly opens Ca^{2+} channels without Na^+ influx. We did not analyze the effect of CBZ on K^+-stimulated neurons, since in kainate-stimulated neurons TTX-sensitive Na^+ channels are also not involved, and because it is clear that VSCCs play an important role.

4.3. Effect of CBZ on $[Na^+]_i$ signals

We also studied the effect of CBZ on the increase in $[Na^+]_i$ stimulated by glutamate ionotropic receptor agonists. As for the $[Ca^{2+}]_i$ measurements, we found that the responses evoked by kainate are mainly mediated by AMPA receptor activation, since they are sensitive to LY 303070, a specific AMPA receptor antagonist (Malva et al., 1998). Moreover, NMDA receptors and voltage-sensitive Na^+ channels are not involved in the increase in the $[Na^+]_i$ caused by kainate, probably because NMDA receptors are blocked by Mg^{2+} and Na^+ channels are inactivated. Thus, in our system and in the situations tested, the main pathway for the influx of Na^+ due to stimulation with kainate are non-NMDA receptors. CBZ did not inhibit the $[Na^+]_i$ signal due to the glutamate ionotropic receptor agonists. This result suggests that CBZ does not inhibit glutamate ionotropic receptor channels, and further supports the effect of CBZ on VSCCs. The drug (400 μM) failed to affect Na^+-dependent binding of [^{3}H]L-glutamate to hippocampal synaptic membranes (Olpe et al., 1985). We also observed that CBZ inhibited the increase in the $[Na^+]_i$ stimulated by veratridine (data not shown), confirming its effects on Na^+ channels, which are not involved in the present stimulation protocol.

Taken together, the present data show that L-type Ca^{2+} channels are the main target of CBZ. These effects may also be responsible for the neuroprotective effects of the drug, in agreement with Lakics et al. (1995), who suggested that mechanisms other than sodium channel blockade may be involved in the neuroprotection. Our results also suggest that glutamate ionotropic receptors are not directly affected by this AED.

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References

Ambrósio, A., Silva, A., Malva, J., Carvalho, C., Soares-da-Silva, P., Carvalho, A., 1998. Effects of carbamazepine on $[Ca^{2+}]_i$ and $[Na^+]_i$ signals stimulated by ionotropic glutamate receptor agonists. Eur. J. Neurosci. 10 (10), 48–54.

Ambrósio, A.F., Malva, J.O., Carvalho, A.P., Carvalho, C.M., 1997. Inhibition of N-, P/Q- and other types of Ca^{2+} channels in rat hippocampal nerve terminals by the adenosine A1 receptor. Eur. J. Pharmacol. 340, 301–310.

Ben-Ari, Y., 1985. Limbic seizures and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14, 375–403.

Berridge, M.J., 1998. Neuronal calcium signaling. Neuron 21, 13–26.

Biber, K., Walden, J., Gebickeharter, P., Berger, M., Vancalker, D., 1996. Carbamazepine inhibits the potentiation by adenosine-analogs of agonist-induced inositolphosphate formation in hippocampal astrocyte cultures. Biol. Psychiatry 40, 563–567.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal TM, a new serum-free medium combination. J. Neurosci. Res. 35, 567–576.

Cai, Z., McCaslin, P.P., 1992. Amitriptyline, desipramine, cyproheptadine and carbamazepine, in concentrations used therapeutically, reduce kainate- and *N*-methyl-D-aspartate-induced intracellular Ca^{2+} levels in neuronal culture. Eur. J. Pharmacol. 219, 53–57.

Chen, G., Pan, B.S., Hawver, D.B., Wright, C.B., Potter, W.Z., 1996. Attenuation of cyclic-AMP production by carbamazepine. J.Neurochem. 67, 2079–2086.

Courtney, M.J., Lambert, J.J., Nicholls, D.G., 1990. The interactions between plasma membrane depolarization and glutamate receptor activation in the regulation of cytoplasmic free calcium in cultured cerebellar granule cells. J. Neurosci. 10, 3873–3879.

Croucher, M.J., Collins, J.F., Meldrum, B.S., 1982. Anticonvulsant action of excitatory amino acid antagonists. Science 216, 899–901.

Dailey, J.W., Reith, M.E.A., Yan, Q.S., Li, M.Y., Jobe, P.C., 1997a. Carbamazepine increases extracellular serotonin concentration—lack of antagonism by tetrodotoxin or zero Ca^{2+}. Eur. J. Pharmacol. 328, 153–162.

Dailey, J.W., Reith, M.E.A., Yan, Q.S., Li, M.Y., Jobe, P.C., 1997b. Anticonvulsant doses of carbamazepine increase hippocampal extracellular serotonin in genetically epilepsy-prone rats–dose response relationships. Neurosci. Lett. 227, 13–16.

Elliott, P., 1990. Action of antiepileptic and anaesthetic drugs on Na- and Ca-spikes in mammalian non-myelinated axons. Eur. J. Pharmacol. 175, 155–163.

Gean, P.W., Huang, C.C., Kuo, J.R., Lin, J.H., Yi, P.L., Tsai, J.J., 1993. Analysis of carbamazepine's anticonvulsant actions in hippocampal and amygdaloid slices of the rat. Chin. J. Physiol. 36, 199–204.

Gelfand, E.W., Cheung, R.K., Grinstein, S., 1986. Mitogen-induced changes in Ca^{2+} permeability are not mediated by voltage-gated potassium channels. J. Biol. Chem. 261, 11520–11523.

Grant, K.A., Snell, L.D., Rogawski, M.A., Thurkauf, A., Tabakoff, B., 1992. Comparison of the effects of the uncompetitive *N*-methyl-D-aspartate antagonist (±)-5-aminocarbonyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine (ADCI) with its structural analogue dizocilpine (MK-801) and carbamazepine on ethanol withdrawal seizures. J. Pharmacol. Exp. Ther. 260, 1017–1022.

Hough, C.J., Irwin, R.P., Gao, X.-M., Rogawski, M.A., Chuang, D.-M., 1996. Carbamazepine inhibition of *N*-methyl-D-aspartate-evoked calcium influx in rat cerebellar granule cells. J. Pharmacol. Exp. Ther. 276, 143–149.

Kapetanovic, I.M., Yonekawa, W.D., Kupferberg, H.J., 1995. The effects of anticonvulsant compounds on 4-aminopyridine-induced de novo synthesis of neurotransmitter aminoacids in rat hippocampus in vitro. Epilepsy Res. 20, 113–120.

Keith, R.A., Mangano, T.J., Salama, A.I., 1989. Inhibition of *N*-methyl-D-aspartate- and kainic acid-induced neurotransmitter release by omega-conotoxin GVIA. Br. J. Pharmacol. 98, 767–772.

Kuo, C.C., Chen, R.S., Lu, L., Chen, R.C., 1997. Carbamazepine inhibition of neuronal Na^+ currents-quantitative distinction from phenytoin and possible therapeutic implications. Mol. Pharmacol. 51, 1077–1083.

Lakics, V., Molnar, P., Erdo, S.L., 1995. Protection against veratridine toxicity in rat cortical cultures—relationship to sodium-channel blockade. NeuroReport 7, 89–92.

Lampe, H., Bigalpe, H., 1990. Carbamazepine blocks NMDA-activated currents in cultured spinal cord neurons. NeuroReport 1, 26–28.

Lancaster, J.M., Davies, J.A., 1992. Carbamazepine inhibits NMDA-induced depolarization in cortical wedges prepared from DBA/2 mice. Experientia 48, 751–753.

Löscher, W., 1998. Pharmacology of glutamate receptor antagonists in the kindling model of epilepsy. Prog. Neurobiol. 54, 721–741.

Lu, Y.M., Yin, H.Z., Chiang, J., Weiss, J.H., 1996. Ca^{2+}-permeable AMPA/kainate and NMDA channels: high rate of Ca^{2+} influx underlies potent induction of injury. J. Neurosci. 16, 5457–5465.

MacDonald, R.L., Kelly, K.M., 1993. Antiepileptic drug mechanisms of action. Epilepsia 34 (5), 51–58.

Malva, J.O., Ambrósio, A.F., Carvalho, A.P., Carvalho, C.M., 1998. AMPA and kainate receptors are active in different regions of the cell membrane in hippocampal neurons. Eur. J. Neurosci. 10 (10), 37–54.

Malva, J.O., Carvalho, A.P., Carvalho, C.M., 1996. Domoic acid induces the release of glutamate in the rat hippocampal CA3 sub-region. NeuroReport 7, 1330–1334.

Mark, R.J., Ashford, J.W., Goodman, Y., Mattson, M.P., 1995. Anticonvulsants attenuate amyloid β-peptide neurotoxicity, Ca^{2+} deregulation and cytoskeletal pathology. Neurobiol. Aging 16, 187–198.

Minato, H., Kikuta, C., Fujitani, B., Masuda, Y., 1997. Protective effect of zonisamide, an antiepileptic drug, against focal cerebral ischemia with middle cerebral artery occlusion-reperfusion in rats. Epilepsia 38, 975–980.

Morimoto, K., Sato, H., Sato, K., Sato, S., Yamada, N., 1997. BW 1003C87, phenytoin and carbamazepine elevate seizure threshold in the rat amygdala-kindling model of epilepsy. Eur. J. Pharmacol. 339, 11–15.

Murphy, S.N., Miller, R.J., 1989. Regulation of Ca^{2+} influx into striatal neurons by kainic acid. J. Pharmacol. Exp. Ther. 249, 184–193.

Okada, M., Kawata, Y., Mizuno, K., Wada, K., Kondo, T., Kaneko, S., 1998. Interaction between Ca^{2+}, K^+, carbamazepine and zonizamide on hippocampal extracellular glutamate monitored with a microdialysis electrode. Br. J. Pharmacol. 124, 1277–1285.

Olpe, H., Kolb, C.N., Hausdorf, A., Haas, H.L., 1991. 4-aminopyridine and barium chloride attenuate the antiepileptic effect of carbamazepine in hippocampal slices. Experientia 47, 254–257.

Olpe, H.-R., Baudry, M., Jones, R.S.G., 1985. Electrophysiological and neurochemical investigations on the action of carbamazepine on the rat hippocampus. Eur. J. Pharmacol. 110, 71–80.

Ozawa, S., 1996. Permeation of Ca^{2+} through glutamate receptor channels. Semin. Neurosci. 8, 261–269.

Phillips, I., Martin, K.F., Thompson, K.S.J., Heal, D.J., 1997. Weak blockade of AMPA receptor-mediated depolarizations in the rat cortical wedge by phenytoin but not lamotrigine or carbamazepine. Eur. J. Pharmacol. 337, 189–195.

Rogawski, M.A., 1995. Excitatory amino acids and seizures. In: Stone, T.W. (Ed.), CNS Neurotransmitters and Neuromodulators-Glutamate. CRC, London, pp. 219–237 Chp. 13.

Rogawski, M.A., Porter, R.J., 1990. Antiepileptic drugs: pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. Pharmacol. Rev. 42, 223–286.

Rush, A.M., Elliott, J.R., 1997. Phenytoin and carbamazepine—differential inhibition of sodium currents in small cells from adult rat dorsal root ganglia. Neurosci. Lett. 226, 95–98.

Sayer, R.J., Brown, A.M., Schwindt, P.C., Crill, W.E., 1993. Calcium currents in acutely isolated human neocortical neurons. J. Neurophysiol. 69, 1596–1606.

Schirrmacher, K., Mayer, A., Walden, J., Dusing, R., Bingmann, D., 1993. Effects of carbamazepine on action potentials and calcium currents in rat spinal ganglion cells in vitro. Neuropsychobiology 27, 176–179.

Schirrmacher, K., Mayer, A., Walden, J., Dusing, R., Bingmann, D., 1995. Effects of carbamazepine on membrane properties of rat sensory spinal ganglion cells in vitro. Eur. Neuropsychopharmacol. 5, 501–507.

Scholz, A., Appel, N., Vogel, W., 1998. Two types of TTX-resistant and one TTX-sensitive Na^+ channel in rat dorsal root ganglion neurons and their blockade by halothane. Eur. J. Neurosci. 10, 2547–2556.

Schumacher, T.B., Beck, H., Steinhäuser, C., Schramm, J., Elger, C.E., 1998. Effects of phenytoin, carbamazepine, and gabapentin on calcium channels in hippocampal granule cells from patients with temporal lobe epilepsy. Epilepsia 39, 355–363.

Sofia, R.D., Gordon, R., Gels, M., Diamantis, W., 1994. Comparative effects of felbamate and other compounds on *N*-methyl-D-aspartic acid-induced convulsions and lethality in mice. Pharmacol. Res. 29, 139–144.

Turski, L., Meldrum, B.S., Turski, W.A., Watkins, J.C., 1987. Evidence that antagonism at non-NMDA receptors results in anticonvulsant action. Eur. J. Pharmacol. 136, 69–73.

Vonwegerer, J., Hesslinger, B., Berger, M., Walden, J., 1997. A calcium antagonistic effect of the new antiepileptic drug lamotrigine. Eur. Neuropsychoparmacol. 7, 77–81.

Waldmeier, P.C., Martin, P., Stocklin, K., Portet, C., Schmutz, M., 1996. Effect of carbamazepine, oxcarbazepine and lamotrigine on the increase in extracellular glutamate elicited by veratridine in rat cortex and striatum. Naunyn Schmiedeberg Arch. Pharmacol. 354, 164–172.

Yokoyama, C.T., Westenbroek, R.E., Hell, J.W., Soong, T.W., Snutch, T.P., Caterall, W.A., 1995. Biochemical properties and subcellular distribution of the neuronal class E calcium channel α_1 subunit. J. Neurosci. 15, 6419–6432.

Yoshida, S., 1994. Tetrodotoxin-resistant sodium channels. Cell. Mol. Neurobiol. 14, 227–244.

Yoshimura, R., Yanagihara, N., Terao, T., Minami, K., Toyohira, Y., Ueno, S., Uezono, Y., Abe, K., Izumi, F., 1998. An active metabolite of carbamazepine, carbamazepine-10,11-epoxide, inhibits ion channel-mediated catecholamine secretion in cultured bovine adrenal medullary cells. Psychopharmacology 135, 368–373.