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The effects of the muscle relaxant, CS-722, on synaptic activity of cultured neurones

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1 The pharmacological properties of the centrally acting muscle relaxant, CS-722, were studied in cultured hippocampal cells and dorsal root ganglion cells of the rat using the whole-cell variation of the patch clamp technique.

2 CS-722 inhibited the occurrence of spontaneous excitatory and inhibitory postsynaptic currents in hippocampal neurones at concentrations of 100-300 μM, but had no effect on postsynaptic currents evoked by the application of glycine, γ-aminobutyric acid, glutamate or N-methyl-D-aspartate.

3 CS-722 reduced voltage-gated sodium currents, while shifting the sodium channel inactivation curve to more negative membrane potentials. This effect is similar to that reported for local anaesthetics. Voltage-gated potassium currents were decreased by CS-722 by approximately 20%, whereas voltage-activated calcium currents were inhibited by about 25%.

4 CS-722 inhibited evoked inhibitory postsynaptic currents. However, the spontaneous quantal release of inhibitory transmitter was not affected.

5 The inhibitory effect of CS-722 on spontaneous inhibitory postsynaptic currents and excitatory postsynaptic currents in hippocampal cultures probably results from an inhibition of both sodium and calcium currents. This inhibitory effect is likely to be amplified in polysynaptic neuronal circuits.

Keywords: Muscle relaxant; hippocampus, sodium currents; calcium currents; glutamate, GABA; anaesthetic

Introduction

Several recent papers have described (**R**)-4-chloro-(2-hydroxy-3-morpholinopropyl)-5-phenyl-4-isoxazolin-3-one hydrochloride (CS-722) as a centrally acting muscle relaxant (Tanabe *et al.*, 1992a,b; 1993). Intravenous injections of CS-722 diminish experimentally produced muscular rigidity in rats, with minimal sedative-like side effects. Other experiments indicate that this drug acts as spinal and supraspinal sites. However, CS-722 does not inhibit synaptic transmission at the neuromuscular junction. CS-722 inhibits both mono- and polysynaptic spinal reflexes, with a significantly greater effect on the latter. Extracellular recordings suggest that CS-722 does not affect pre- or postsynaptic potentials evoked in the spinal dorsal horn, but it does reduce the electrical excitability of primary afferent fibres and motoneurones.

These previous studies led to the conclusion that CS-722 suppresses general neuronal excitability by a 'membrane stabilizing action', thereby increasing the threshold of neuronal firing in the polysynaptic spinal circuit. We have assessed the effects of CS-722 in a neuronal network of spontaneously active cultured hippocampal neurones of the rat using the whole-cell patch clamp technique. An attempt was made to correlate the observed effects of CS-722 on spontaneous activity to a modification of one or more types of ligand- and voltage-gated currents recorded from these same cells. In a few experiments, dorsal root ganglion (DRG) neurones were also tested. We offer experimental evidence that CS-722 has a weak local anaesthetic effect and propose that sodium channel inhibition reduces overall synaptic excitability. We further demonstrate a CS-722-induced inhibition of calcium currents and postulate that this action could reduce presynaptic transmitter release. Both effects contribute to the overall inhibition of spontaneous hippocampal synaptic activity by CS-722.

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Methods

Preparation of cells

Hippocampal neurones were prepared from 17-day embryonic Sprague-Dawley rat pups by a technique detailed elsewhere (Marszalec & Narahashi, 1993). The final cell suspension was plated onto 12 mm poly-L-lysine coated coverslips having a confluent layer of glia plated beforehand. The glial/hippocampal co-culture was maintained in a humidified atmosphere of air and 10% CO_2 at 37°C. Hippocampal neurones develop an increasing sensitivity to γ-aminobutyric acid (GABA) and glutamate during the first week in culture as well as discernible cell to cell axonal contacts (see Köller *et al.*, 1990). After 7 days, discrete spikes or bursts of spontaneously occurring excitatory and inhibitory postsynaptic currents could be recorded. The relative amount or spontaneous excitatory or inhibitory current varied from cell to cell. All recordings were made from pyramidal-shaped cells having triangular soma (30-50 μm) and multipolar dendritic trees. Smaller bipolar neurones were also observed, but were not recorded from. The presence of dentate gyrus granule cells is reported to be minimal at the embryonic age of the animals used (Banker & Goslin, 1991).

Acutely dissociated hippocampal cells were isolated by a technique adapted from Kay & Wong (1986) and described by Song & Narahashi (1995). The hippocampus was isolated from 7-14 day postnatal rat pups, sectioned into 500 μm slices, and trypsinized in a stirring chamber. After dissociation, neurones were allowed to settle on poly-L-lysine-coated coverslips. These were used for experiments 1-7 h after plating.

DRG neurones were isolated as previously described (Roy *et al.*, 1994). Spinal ganglia isolated from 2-7 day postnatal rat pups were plated on poly-L-lysine-coated coverslips. These cells were incubated in air and 10% CO_2 at 37°C for 2-7 h before experimental use.

Electrophysiological recordings

Whole-cell currents were recorded by the techniques of Hamill *et al.* (1981). Recording electrodes were pulled from borosilicate glass (Kimble, Vineland, NJ, U.S.A.) on a two-step

vertical puller (Narishige, Tokyo, Japan) to a final resistance of 1.5 to 2.0 MΩ when filled with internal solution. Most currents were recorded with an Axopatch-1C patch clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). These were filtered at 2 kHz and digitized by a PC-based data acquisition system that also permitted preliminary data analysis.

Sodium channel currents were recorded with an Axopatch 200 amplifier (Axon Instruments). These currents were digitized and analysed using a PDP 11/73 computer (Digital Equipment Corp., Pittsburgh, PA). Capacitive and leakage currents were subtracted by the P + P/4 procedure (Bezanilla & Armstrong, 1977).

Evoked synaptic potentials were recorded in some experiments from isolated pairs of neurones having observable axonal/dendritic contacts and intersomal distances of 200 μm or greater. A glass electrode (1–2 MΩ, filled with extracellular solution) was positioned against the soma of the presynaptic cell. A 0.1–1 ms voltage pulse of 5–10 V delivered from a step generator evoked synaptic currents in the postsynaptic cell which were recorded via the patch clamp electrode.

All experiments were performed at room temperatures (22–25°C).

Solutions

The standard external solution used for spontaneous activity and ligand-gated recordings contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2.5, HEPES-acid 5.5, HEPES-Na^+ 4.5, glucose 10, and pH adjusted to 7.3 with NaOH. The standard internal solution consisted of (in mM): CsCl 140, $MgCl_2$ 2.0, $CaCl_2$ 1, EGTA 11, HEPES-acid 10, Mg-ATP 11, and pH adjusted to 7.3 with CsOH. When recording spontaneous hippocampal activity the CsCl content of the internal solution was replaced by K-gluconate.

Potassium current recordings utilized the same standard internal solution as above, except for the replacement of CsCl by KCl. The external solution also contained 0.4 μM tetrodotoxin (TTX) and 50 μM $CdCl_2$ to block sodium and calcium currents, respectively. Calcium currents were recorded with external solutions consisting of (in mM): NaCl 150, $BaCl_2$ 5, $MgCl_2$ 2, HEPES 10, glucose 10, 0.4 μM TTX, and pH adjusted with NaOH to 7.3. The internal solution contained (in mM): CsCl 150, $MgCl_2$ 2, EGTA 10, HEPES 10, Mg^{2+}-ATP 4, Na^+-GTP 0.1, and pH adjusted to 7.2 with CsOH.

Sodium channel currents were recorded with an external solution of (in mM): NaCl 25, tetramethylammonium (TMA) chloride 75, tetraethylammonium (TEA) chloride 20, CsCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 25, HEPES-acid 5, and pH adjusted to 7.4 with TEA-OH. $LaCl_3$ (3 μM) was also present to inhibit calcium currents. The pipette solution contained (in mM): CsF 135, NaCl 10, HEPES-acid 5, and the pH was adjusted to 7.0 with CsOH.

Drugs

Ligand-gated channel activation was achieved by the use of a computer-operated U-tube drug delivery system (Marszalec & Narahashi, 1993) that permitted localized solution exchanges within 15–20 ms. The following compounds were all from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); L-glutamate, GABA, glycine, and bicuculline methiodide. N-methyl-D-aspartic acid (NMDA), baclofen, and 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). CS-722 was a gift from Sankyo Co. Ltd. (Tokyo, Japan).

Results

Effects of CS-722 on spontaneous activity

Cultured hippocampal neurones develop synaptic connections and accompanying spontaneous activity after 3–5 days in culture (Köller *et al.*, 1990; Siebler *et al.*, 1993). The block of sodium channels by TTX or of synaptic transmission by magnesium inhibits such spontaneous activity, implying underlying action potential formation and transmitter release. One study (Köller *et al.*, 1990) suggests that this activity is initiated by a spontaneous vesicular release of glutamate from presynaptic terminals, depolarizing postsynaptic cells to the action potential threshold. The propagation of action potentials to nerve terminals evokes further transmitter release, thus activating excitatory and inhibitory synapses throughout the neuronal network.

The control records in Figure 1 show spontaneous hippocampal currents as recorded with the whole-cell patch clamp technique. Here, the standard chloride concentration of the patch pipette was largely replaced with the gluconate anion. At membrane potentials of −40 to −50 mV inhibitory postsynaptic currents (i.p.s.cs) and excitatory postsynaptic currents (e.p.s.cs) could be differentiated. Outward-going i.p.s.cs are depicted in the record as upward events, whereas inward-going e.p.s.cs are downward. No action potentials are observed in the patch clamped neurone at these holding potentials. The application of the glutamate antagonist, CNQX, abolished both e.p.s.cs and i.p.s.cs (Figure 1b). This is to be expected as the glutamate receptor antagonism blocks the excitation of both glutamate and GABAergic presynaptic neurones making synaptic contacts with the recorded cell. Figure 1d shows the inhibition of the upward i.p.s.cs by the $GABA_A$ antagonist, bicuculline. The presence of bicuculline also increased the frequency of e.p.s.cs.

The current recordings in Figure 2a and b show a typical effect of a 3 min bath perfusion of CS-722 (300 μm). In most instances, CS-722 reduced both i.p.s.c. and e.p.s.c. frequency, with the latter usually being inhibited to a greater degree ($n=6$). It should be noted, however, that in two experiments

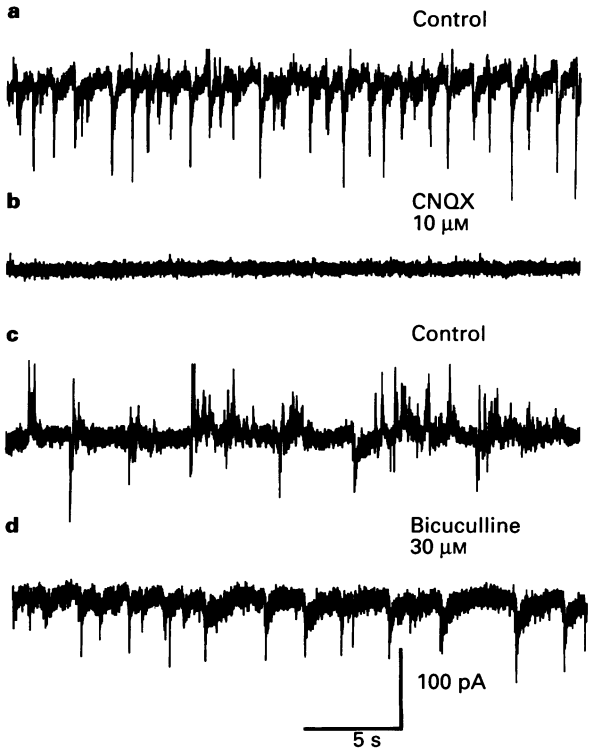

Figure 1 Spontaneous postsynaptic currents recorded from pyramidal neurones cultured for 8 days. The recording pipette contained K^+ gluconate (140 mM) producing upward i.p.s.cs and downward e.p.s.cs at a holding potential of −40 mV. (a) Control; (b) block of both e.p.s.cs and i.p.s.cs by CNQX, (10 μM) an excitatory amino acid receptor antagonist; (c) control from a different neurone; (d) bicuculline 30 μM, a $GABA_A$ receptor antagonist, suppressed i.p.s.cs and increased the frequency of e.p.s.cs.

CS-722 increased the frequency of i.p.s.cs (see Figure 6). The CS-722 effect always reversed upon washout with drug-free solution. The depression of spontaneous activity observed with a direct U-tube application of 300 μM CS-722 (Figure 2c) was nearly instantaneous, as was its reversal. The expanded scale of Figure 2d indicates that CS-722 often converts repetitive 'bursts' of i.p.s.cs and e.p.s.cs into single events.

Figure 3 shows the dose-dependence of the CS-722 response with minimal, moderate, and maximal effects being observed with 30 μM, 100 μM, and 300 μM CS-722, respectively.

Effects of CS-722 on postsynaptic ligand-activated currents

Ligand-gated currents could be evoked in cultured hippocampal neurones (in the presence of TTX, 0.4 μM, to block spontaneous activity) by the direct application of GABA, glycine, glutamate or NMDA. Figure 4 shows the effect of CS-722 on these ligand-gated responses. In all experiments CS-722 was bath perfused 3 min prior to its co-application with a given agonist. These experiments clearly show that neither high (300 μM) nor low (100 μM) concentrations of CS-722 modified the current responses produced by any of these agonists.

Effects of CS-722 on sodium currents

Voltage-gated sodium currents were studied in both rat DRG neurones and acutely dissociated rat hippocampal cells. Figure 5 shows sodium current traces recorded from the hippocampal cell preparation. The bath application of CS-722 (300 μM) reversibly decreased the peak sodium current evoked by a 5 ms

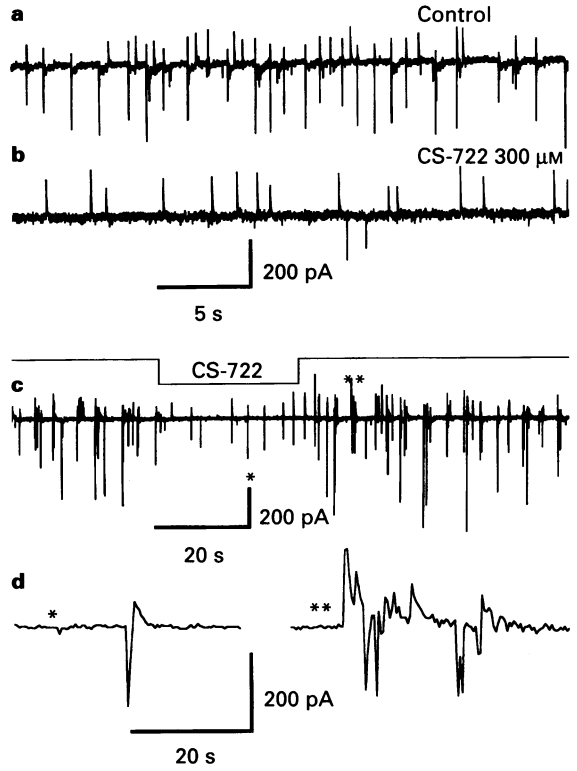

Figure 2 Inhibition of spontaneous activity in cultured hippocampal cells by the muscle relaxant, CS-722. (a and b) Spontaneous i.p.s.cs and e.p.s.cs recorded over a 30 s recording period. Recordings were made with pipettes containing K^+ gluconate (140 mM) at a holding potential of −50 mV producing upward i.p.s.cs and downward e.p.s.cs. Coupled e.p.s.cs and i.p.s.cs probably arise from the simultaneous activation of both glutamate synapses at the recorded cell and presynaptic inhibitory neurones synapsing with it. The bath perfusion of CS-722 300 μM, (b) reduced the number of e.p.s.ps by 93%, while i.p.s.cs were inhibited by 52%. The effects of CS-722 reversed upon washout. (c and d) The direct U-tube application of CS-722 (300 μM) inhibited e.p.s.cs and i.p.s.cs recorded at a holding potential of −50 mV with a rapid onset and reversal. The asterisked events in (c) are expanded in (d) to show that CS-722 sometimes converts bursts of e.p.s.cs and i.p.s.cs into single events.

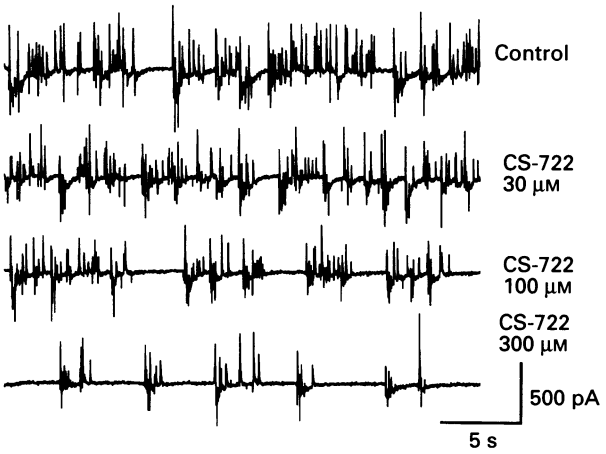

Figure 3 Dose-dependence of the effect of CS-722 on a spontaneously active hippocampal cell (holding potential; −50 mV). The numbers of e.p.s.cs (downward) and i.p.s.cs (upward) progressively decreased as the bath concentration of CS-722 was raised. At CS-722 concentrations of 30, 100, and 300 μM, e.p.s.c occurrence per min decreased by 0, 32, and 84% respectively. Similarly, i.p.s.cs per min declined by 0, 22, and 67% respectively.

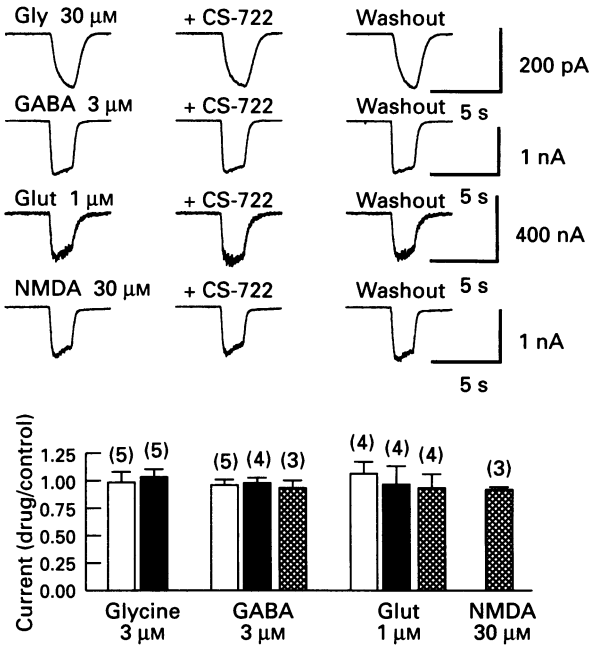

Figure 4 CS-722 does not affect postsynaptic currents evoked by the application of several ligands. Currents were evoked in cultured hippocampal neurones by 2 s U-tube applications of glycine, GABA, glutamate or NMDA. The holding potential in all experiments was −60 mV. Spontaneous activity was inhibited by TTX, 0.4 μM. The internal solution used here produced inward currents with every agonist tested. In the middle column of each series, the agonist and CS-722 were co-applied following a 3 min bath perfusion of CS-722 alone. The CS-722 concentration used was 300 μM, except in the glycine experiment where it was 100 μM. NMDA was co-applied with 0.6 μM gylcine, the NMDA receptor co-agonist. The results of all experiments are summarized in the lower graph, which indicates the means (±s.e. mean) of current amplitudes recorded in the presence of CS-722 as values relative to those of the control; CS-722 30 μM, open columns; 100 μM, solid columns; 300 μM, cross hatched columns. The numbers in parenthesis indicate the number of cells tested. No significant effect of CS-722 was observed for any agonist.

voltage step from −80 mV to 0 mV (Figure 5a). The second set of traces indicates that CS-722-induced inhibition was nearly doubled at a holding potential of −60 mV. Figure 5(b) shows the effect of CS-722 on the sodium channel inactivation (h_∞) curve. Currents evoked by step depolarizations to 0 mV from various holding potentials were normalized to the maximum response recorded from a holding potential of −100 mV. The sodium channel inactivation curve in the presence of CS-722 was shifted in the hyperpolarizing direction. This could be interpreted to mean that CS-722 has a higher affinity for channels in the inactivated state (Hille, 1992). The CS-722-induced shift of the sodium inactivation curve did not completely reverse after washout.

It should be noted that CS-722 did not shift the sodium channel activation curve (not shown). Therefore, CS-722 did not alter the voltage-sensitivity of channel gating.

The sodium currents of DRG neurones were similarly affected by CS-722 with a reduction of the peak response along with a shift of the inactivation curve (data not shown).

Effects of lignocaine on spontaneous activity

Sodium current inhibition accompanied by a shift of the sodium inactivation curve is a characteristic of local anaesthetics (Courtney, 1975; Hille, 1975; 1992). Therefore, the effects of lignocaine on spontaneously active cultured hippocampal neurones were compared with CS-722 (Figure 6). A perfusion of lignocaine (30 μM) or CS-722 (300 μM) produced identical decreases of e.p.s.c. frequency. Surprisingly, in this cell, both drugs generated i.p.s.cs not present in the control or after washout. The mechanism of this potentiation of inhibitory activity is unclear, although it may arise from some complex disinhibition of GABAergic cells within the neuronal network. More significant was the observation that the effects of CS-722 on spontaneous hippocampal activity were mirrored by the local anaesthetic.

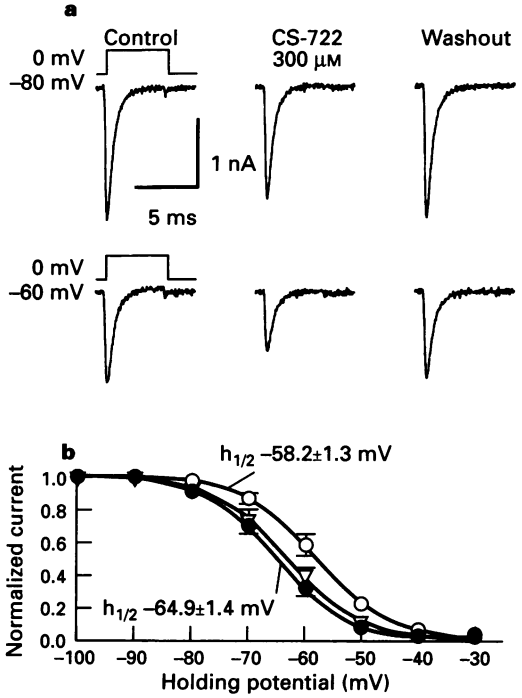

Figure 5 CS-722 at 300 μM inhibits sodium currents in acutely dissociated hippocampal cells. Sodium currents were evoked by applying 5 ms step depolarizations from various holding potentials to 0 mV. (a) The two sets of traces show that CS-722 inhibition is greater when the holding potential is less negative. (b) The steady-state inactivation curves (mean ± s.e. mean, $n=4$); currents were evoked by voltage steps to 0 mV following 20 s conditioning potentials as indicated on the abscissa scale. All currents are normalized to the maximal response at the −100 mV conditioning potential of each experimental set. (○) Control; (●) CS-722 300 μM; (▽) wash. $h_{1/2}$ indicates the conditioning potential that produces a 50% maximal current. The inactivation curve is shifted by CS-722 in the hyperpolarizing direction, and the recovery after the washout is very small.

Effects of CS-722 on potassium and calcium currents

In some voltage-clamped cells, the bath perfusion of CS-722 (300 μM) generated a net inward current of 50–100 pA at a holding potential of −40 mV (Figure 7a). This was more often observed in cells where potassium was present as the primary cation of the pipette solution. This current reversed direction near −80 mV, suggesting that it might result from an inhibition of a steady outward potassium current. Therefore, the effects of CS-722 were tested on voltage-gated potassium currents recorded from cultured DRG and hippocampal neurones. Figure 7b shows potassium currents generated in a cultured hippocampal cell by step depolarizations from −80 to 0 mV. The response consists of an initial peak that levels off to a smaller steady-state phase. A 3 min bath perfusion of CS-722 (300 μM) reduced peak and steady-state potassium currents by an average of 17.2 ± 2.2% and 19.4 ± 2.7%, respectively ($n=5$). A similar inhibition was also observed in dorsal root ganglion cells. Thus, CS-722 reversibly inhibited both phases of the potassium current.

The effects of CS-722 were also tested on voltage-gated calcium currents generated by step depolarizations from holding potentials of −80 mV to 0 mV in cultured hippocampal neurones (Figure 7c). A bath perfusion of 300 μM CS-722 reduced the average peak calcium current amplitude by 23.7 ± 8.4% ($n=6$). However, the current-voltage relationship for the calcium current was not shifted by CS-722 along the voltage axis (data not shown). Interestingly, in two of three dorsal root ganglion cells tested, the effect of CS-722 on calcium currents was negligible.

Effect of CS-722 on synaptically-evoked responses

Experiments were undertaken to test whether CS-722 altered presynaptic transmitter release. Synaptic postsynaptic responses were evoked from cultured hippocampal neurones by stimulating presynaptic cells with localized extracellular electrical current. Spontaneous activity was reduced by adding

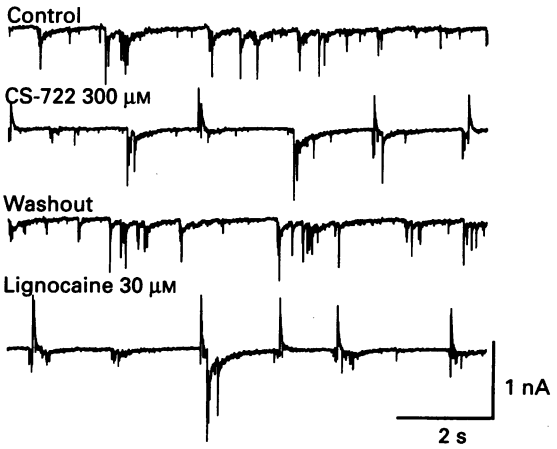

Figure 6 The local anaesthetic, lignocaine, mimics the effect of CS-722 on spontaneous hippocampal activity. The top two traces show 10 s recordings of spontaneous activity in the absence and presence of CS-722 (300 μM). Here, the usual e.p.s.c. (downward) reduction was observed. However, in this cell an increased incidence of i.p.s.cs (upward) was observed in the presence of CS-722. The perfusion of 30 μM lignocaine affected the spontaneous activity in the same manner as did CS-722, suggesting that CS-722 produces a local anaesthetic-like effect.

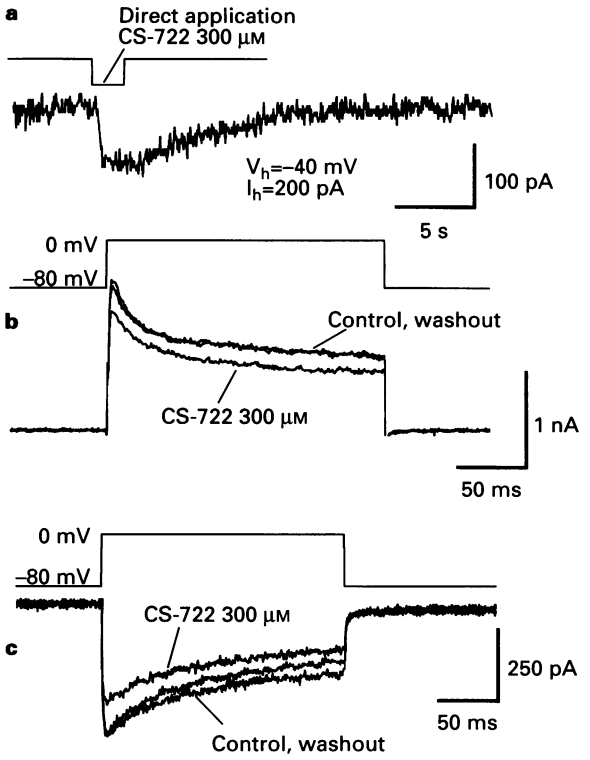

Figure 7 The effects of CS-722 on voltage-gated potassium and calcium currents. (a) The net inward current evoked by the direct U-tube application of CS-722 (300 μM) to a dorsal root ganglion cell. This current reversed near −80 mV, the approximate equilibrium potential for potassium. (b) Potassium currents evoked in a cultured hippocampal neurone by a step depolarization from a holding potential of −80 mV to 0 mV. The bath perfusion of CS-722 (300 μM) reversibly inhibited both the peak and steady-state phases of the current by 20%. (c) The effect of CS-722 on voltage-gated calcium currents also evoked in a cultured hippocampal cell. Currents were evoked by 200 ms step depolarizations from −80 to 0 mV. In the presence of CS-722 (300 μM), the peak current was reduced by 22%, and the inhibition was reversible upon washout.

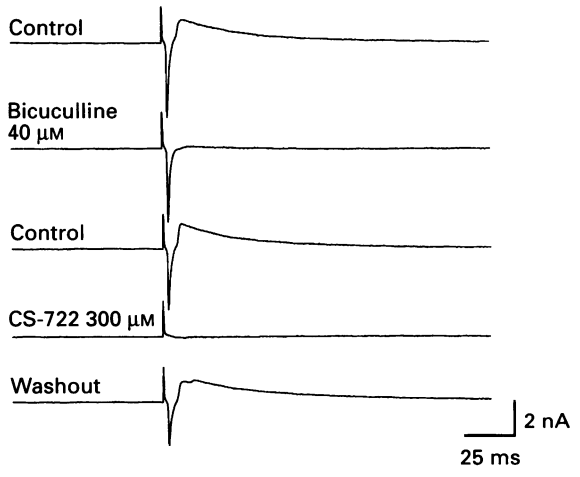

Figure 8 The effects of CS-722 on evoked i.p.s.cs recorded from a cultured postsynaptic hippocampal cell in response to an external electrical stimulus (100 μV, 100 μs) applied to a presynaptic cell. The postsynaptic holding potential is −50 mV. The initial upward-spike in each trace represents a stimulus artifact. The subsequent inward-response is a TTX-sensitive sodium current directly activated in the postsynaptic cell by the electric stimulus. The upward current is the i.p.s.c. evoked by the presynaptic release of GABA. This notion is supported by the suppression of this i.p.s.c. by the $GABA_A$ antagonist bicuculline. A 3 min perfusion of CS-722 (300 μM) abolished both the sodium current and the i.p.s.c Each current component returned upon CS-722 washout.

3 mM Mg^{2+} to the external solution. In four cells, the stimulus evoked a postsynaptic response consisting of an inward TTX-sensitive sodium current and an outward i.p.s.c. (Figure 8). The stimulation is marked by the initial upward stimulus artifact found in each record. No evoked e.p.s.cs were observed, nor was any current component affected by the application of the glutamate antagonist, CNQX. However, i.p.s.cs were inhibited by the $GABA_A$ receptor antagonist, bicuculline (Figure 8) and by the $GABA_A$ receptor agonist, baclofen (record not shown). The postsynaptic sodium current response may have resulted from the direct electrical stimulation of sodium channels in the postsynaptic membrane (Scholtz & Miller, 1991). In three cells, CS-722 (300 μM) inhibited both the i.p.s.c. and the sodium current. This inhibition reversed upon CS-722 washout. In the fourth cell, CS-722 affected neither response.

Effects of CS-722 on quantal transmitter release

Several studies report that some drugs that inhibit the evoked synaptic release of transmitter can also decrease spontaneous non-synaptic quantal transmitter release (Scholz & Miller, 1992; Scanziani *et al.*, 1992; Thompson *et al.*, 1993). The effects of CS-722 on quantal release in cultured hippocampal cells were studied in the presence of TTX (0.4 μM) to block all action potential-generated activity. Under these conditions, small miniature(m)-i.p.s.cs were observed in some hippocampal cells.

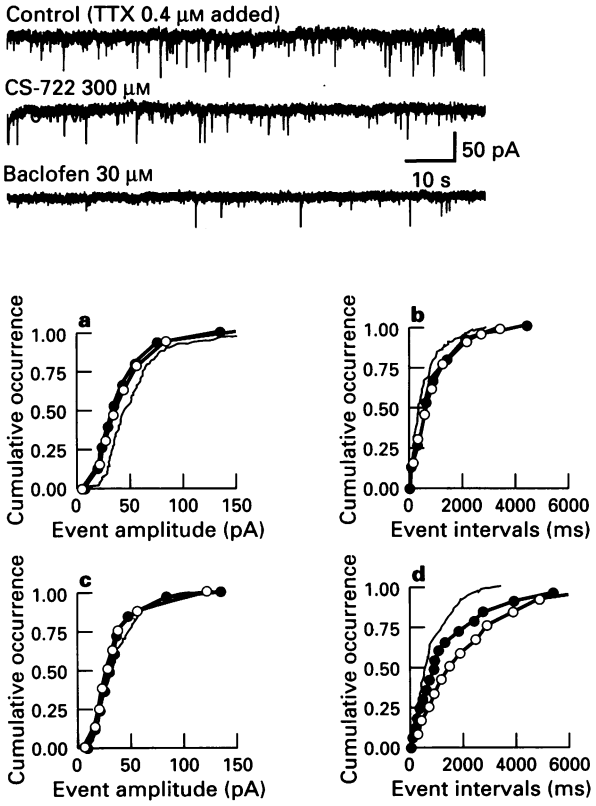

Figure 9 CS-722 is ineffective in preventing spontaneous quantal transmitter release. The top three traces are 1 min recordings from a single hippocampal cell in the presence of TTX (0.4 μM) to inhibit all sodium channel-mediated activity. The small inward responses are bicuculline-sensitive miniature (m)-i.p.s.cs produced by the spontaneous release of GABA from presynaptic terminals. m-i.p.s.c. records collected for 4 min periods were used to generate graphs of m-i.p.s.c. amplitude distributions (a and c) and frequency distributions (b and d). CS-722 (300 μM, ○) had no effect on either m-i.p.s.c. amplitude (a) or frequency (b). Baclofen (30 μM, ○) did not change the distribution of m-i.p.s.c. amplitudes (c), but shifted the distribution of inter-event intervals (d) to longer times (i.e. baclofen decreased the frequency of m-i.p.s.cs). In (a), (b), (c) and (d) (●) wash; third tracing (no symbols), control.

m-i.p.s.cs arise from the action potential-independent quantal release of GABA from presynaptic vesicles and were much smaller than true synaptically-evoked i.p.s.cs. Examples of the bicuculline-sensitive m-i.p.s.cs are shown in the three current traces at the top of Figure 9. Here, the perfusion of the $GABA_B$ agonist, baclofen, significantly reduced the frequency, but not the m-i.p.s.cs amplitude (Figure 9c and d). This type of inhibition has been reported for compounds identified as being $GABA_B$, μ opioid and adenosine A_1 agonists. However, CS-722 applied to the same cell produced no change of m-i.p.s.c. frequency or amplitude (Figure 9a and b). This suggests that CS-722 is ineffective in inhibiting non-synaptic quantal release. Since we were unable to observe m-e.p.s.cs, the effect of CS-722 on these events could not be investigated.

Discussion

The bath perfusion of 100–300 μM CS-722 decreases the frequency of spontaneous e.p.s.cs recorded from cultured hippocampal neurones in the absence of TTX. In most cells, i.p.s.c. frequency is also reduced, but in a few neurones CS-722 seems to enhance their appearance. CS-722 also converts repetitive 'bursts' of e.p.s.cs and i.p.s.cs to single events.

One possible explanation for these observations is a selective interaction between CS-722 and a specific postsynaptic receptor channel. Such interactions have been reported for the potentiation of $GABA_A$ receptors channels by many benzodiazepine agonists (Macdonald & Olsen, 1994) and for the inhibition of non-NMDA glutamate channels by another benzodiazepine, GYKI 52466 (Donevan & Rogawski, 1993). Both drugs have been shown to have muscle relaxant actions (Biscoe & Fry, 1982; Davidoff, 1985; Block & Schwarz, 1994). However, CS-722 clearly does not modify hippocampal currents evoked by glycine, GABA, glutamate or NMDA in hippocampal neurones.

An alternative mechanism of CS-722-induced inhibition could arise from an action on either voltage-activated potassium or calcium currents. Such an effect might occur via a direct interaction of CS-722 with channels or by an indirect action similar to that described for $GABA_B$, μ-opioid, and adenosine A_1 agonists (Andrade *et al.*, 1986; Trussell & Jackson, 1987; Dutar & Nicoll, 1988; Scholz & Miller, 1991). Receptor binding by these latter agonists modify potassium or calcium currents at both pre- and postsynaptic sites by a G-protein mediated process.

CS-722 decreases voltage-gated potassium currents in cultured hippocampal and DRG cells by about 20%. It also seems to inhibit an outward steady-state potassium current at membrane potentials near −50 mV. An inhibition of potassium channels would tend to increase neuronal excitability and possibly enhance synaptic transmission. This, however, is in contrast to the depressant effect on spontaneous hippocampal activity observed with CS-722.

Calcium currents are also inhibited in the presence of 300 μM CS-722 by approximately 25%. This effect is compatible with the suppression of both spontaneous and evoked synaptic currents produced by CS-722. This effect could arise from an inhibition of calcium currents at presynaptic terminals, producing a subsequent reduction of calcium-dependent transmitter release. The action of CS-722 was similar to baclofen and adenosine, two compounds believed to reduce evoked synaptic responses by an inhibition of calcium current (Zhu & Ikeda, 1993; Umemia & Berger, 1994; Doze *et al.*, 1995; Huston *et al.*, 1995). CS-722, however, had no effect on spontaneous quantal transmitter release as reported for these other two agents (Scholz & Miller *et al.*, 1992; Scanziani *et al.*, 1992; Thompson *et al.*, 1993).

CS-722 inhibits sodium currents in hippocampal and DRG neurones in a voltage-dependent manner. It also shifts the sodium channel inactivation curve to more negative potentials. A similar dual effect has been reported for the action of local anaesthetics (Hille, 1992). In the present study, a perfusion of 30 μM lignocaine modified spontaneous hippocampal activity in a manner indistinguishable from that of CS-722. Therefore, it is likely that the mechanism of spontaneous and evoked synaptic current inhibition by CS-722 results, in part, from the inhibition of sodium currents.

CS-722-induced inhibition of sodium current in the cultured hippocampal preparation could reduce overall membrane excitability and inhibit transmitter release. Local anaesthetics penetrate nerves and block sodium channels from the cytoplasmic side of the membrane (Narahashi *et al.*, 1970; Frazier *et al.*, 1970). The larger surface to volume ratio of small nerve fibres over larger fibres promotes a faster channel block in smaller fibres than in larger ones (Narahashi, 1994). At small diameter nerve terminals this channel block may disrupt action potential propagation and inhibit the release of transmitter, thereby suppressing e.p.s.cs and i.p.s.cs. Such reduction of transmitter release has been demonstrated in rat olfactory neurones in the presence of lignocaine (Iwasaki, 1989). The block of sodium channels in cultured hippocampal neurones by TTX inhibits all spontaneously-occurring e.p.s.ps and i.p.s.ps. Presumably, a similar (but less potent) block produced by CS-722 produces a comparable effect.

It is possible that CS-722-induced muscle relaxation develops from a combined sodium and calcium channel block that elevates action potential thresholds, reduces axonal/dendritic signal propagation, and decreases transmitter release. The effect of these individual events could increase in proportion to the number of neurones involved in the synaptic circuit. Thus, the inhibitory influence of CS-722 on the overall activity of neuronal networks like those found in the spinal cord and hippocampal culture may be greater than that observed on any single cell.

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