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Losigamone decreases spontaneous synaptic activity in cultured hippocampal neurons

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Abstract

Losigamone is a new antiepileptic drug with an unknown mechanism of action. Here we report on the effects of losigamone on the synaptic activity in a network of cultured rat hippocampal neurons. Losigamone dose dependently reduced the frequency of spontaneous synaptic events without affecting the mean current amplitude. The drug affected equally the isolated inhibitory as well as excitatory postsynaptic currents. Miniature postsynaptic currents were not altered by losigamone, suggesting that the mechanism of action depends on functional Na^+ channels. Consistent with these findings, the drug decreased the frequency of spontaneous action potentials and suppressed repetitive firing of neurons. Thus, losigamone generally depresses synaptic activity in a neuronal network without selectively modulating a specific postsynaptic receptor type. We conclude that losigamone acts via a presynaptic mechanism reducing neuronal excitability.

Keywords: Epilepsy; Losigamone; Cell culture; IPSC (inhibitory postsynaptic current); EPSC (excitatory postsynaptic current)

1. Introduction

Many patients with epileptic syndromes cannot be treated adequately with the presently available anticonvulsive drugs. Therefore, new agents with different mechanisms of action are required. The tetronic acid derivative, (±)-(5*RS*,*aSR*)-5-[(2-chlorophenyl)hydroxymethyl]-4-methoxy-2(5*H*)-furanone (losigamone), is a new substance with good efficacy against experimentally induced seizures in rats and mice (Zhang et al., 1992; Nöldner and Chatterjee, 1990) and in various in vitro models (Köhr and Heinemann, 1990a,b; Zhang et al., 1992; Yonekawa et al., 1995). Losigamone is presently being tested in controlled clinical trials (Runge et al., 1993). The drug is not structurally related to any other established anticonvulsant and the mechanism of action remains unknown. Recent data suggest that losigamone enhances the GABA (γ-amino-butyric acid)$_A$ receptor-mediated Cl^- influx into spinal cord neurons (Dimpfel et al., 1995), although a direct interaction with the known modulatory binding sites on $GABA_A$ receptors could not be demonstrated. Recordings from CA1 and entorhinal cortex neurons have shown that losigamone has only little effect on postsynaptic potentials but lowers excitability by suppression of repetitive spike firing (Schmitz et al., 1995). Thus, it is still unknown whether the drug reduces excitability by changing the intrinsic properties of neurons or by altering postsynaptic responses.

We have now studied the effects of losigamone on synaptic activity in cultured rat hippocampal neurons, using patch-clamp techniques. This preparation provides a network of synaptically connected neurons displaying spontaneous excitatory and inhibitory transmission. In this system, any postsynaptic modulation of the $GABA_A$ receptor should be detectable from alterations of the amplitude or decay time course of the inhibitory postsynaptic currents (IPSCs; Mody et al., 1994) whereas a presynaptic mechanism of action would preferentially affect the frequency of events.

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2. Materials and methods

2.1. Cell culture

Hippocampal cell cultures were prepared from Wistar rat embryos at embryonic day 18 according to the protocol of Banker and Cowan (1977). Briefly, the pregnant rat was decapitated under deep ether anaesthesia, the embryos removed and killed by cervical dislocation. The brains were taken out and cooled until the hippocampi were dissected. Neurons were triturated and plated on poly-D-lysine-coated culture dishes (Falcon, Heidelberg, Germany, 35 mm) or on coverslips at a density of 500 000 cells per dish (50 000 cells per coverslip) in 1 ml BME (basal medium, Eagle) medium (Gibco, Karlsruhe, Germany) supplemented with 2 mM glutamine, 10% glucose and 10% horse serum (Gibco). Neuronal cultures were maintained at 37°C in 5% CO_2 until used for experiments.

2.2. Electrophysiological recordings

Patch-clamp recordings were performed at room temperature (20–24°C) using cells cultured for 10–30 days. In most cases the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to record spontaneous synaptic currents at −60 mV holding potential. Only extracellular recordings of action potentials were obtained in the cell-attached configuration both in the voltage- and the current-clamp mode. Patch-clamp electrodes of 2–5 MΩ pipette-to-bath resistance were fabricated from borosilicate glass (Hilgenberg, Malsfeld, Germany; outer diameter 2 mm, inner diameter 1 mm). Pipettes were filled with an intracellular solution containing (in mM): KCl, 120; $CaCl_2$, 1; $MgCl_2$, 2; EGTA, 11; HEPES, 10; glucose, 20. The extracellular solution consisted of (in mM): NaCl, 130; KCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 25. The pH of both solutions was adjusted to 7.3. Currents were recorded with a standard patch-clamp amplifier (EPC-7, List Medical Instruments, Darmstadt, Germany), filtered at 3 kHz and stored on video tape for off-line analysis. For stimulated synaptic currents, a second patch pipette was positioned at a neighboring cell and square-voltage pulses of 0.2 ms duration were applied at variable amplitude until a stable stimulation response was reached.

Losigamone was a kind gift of Dr. Willmar Schwabe GmbH (Karlsruhe, Germany). The substance was dissolved at 500 mM in DMSO (dimethyl sulfoxide) and subsequently diluted in the extracellular solution to the final concentration of ≤ 100 μM. At this concentration ($\leq 0.02\%$) DMSO had no effect on the frequency, amplitude or kinetics of postsynaptic currents ($n = 5$ cells). All other substances were purchased from Sigma (Deisenhofen, Germany). NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo-quinoxaline-7-sulfonamide) was dissolved in DMSO, whereas all other drugs were dissolved in water.

2.3. Data analysis

Current traces of 30–60 s duration were analysed offline from video tape. The signal was low-pass filtered at 1 kHz, redigitized at 3 kHz with a CED1401 interface (CED, Cambridge, UK) and fed into the CDR automatic event detection program written by Dr. J. Dempster, University of Strathclyde, Scotland, UK. For macroscopic spontaneous currents the threshold for event detection was set to −20 pA from the running baseline, which was calculated from 1.5 ms long data stretches. Miniature postsynaptic currents could be detected reliably by setting the threshold at about twice the noise amplitude below the baseline. These parameters were developed by comparison with hand-evaluated data stretches and allowed for a detection of all synaptic events which were clearly separated from baseline noise. The shortest possible interval between two successive events was set to 2.5 ms. Thus, events within bursts of repetitive synaptic currents were included in the analysis and counted from the running baseline, i.e., as net increase in current amplitude (cf., Salin and Prince, 1996). The currents were then further analysed within the SCAN subprogram of the same software package. We counted the frequency and amplitude of the postsynaptic currents before, during and after application of the respective dose of losigamone. For the analysis of current decay kinetics, we selected isolated events and discarded traces with multiple overlapping events. Decay time constants were computed by fitting bi-exponential functions to the decaying part of individual current traces within the TIDA program (HEKA, Lambrecht, Germany). The resulting parameters were subsequently averaged for each cell. Stimulated IPSCs were likewise analysed within TIDA.

The statistical significance of drug effects was evaluated by comparison of data obtained before and after application of the drug. We used the non-parametric Wilcoxon test for this purpose. Amplitude distributions were compared using the Kolmogorov-Smirnov test for the cumulative probability distribution before and after application of losigamone (Cohen et al., 1992; Salin and Prince, 1996). $P < 0.05$ is regarded as significant.

3. Results

3.1. Spontaneous synaptic activity

Whole-cell currents were recorded from 57 cultured hippocampal neurons with an input resistance of 477 ± 283 MΩ. After more than 7 days in vitro the neurons displayed spontaneous postsynaptic currents which were measured at −60 mV holding potential. The frequency of these events varied between cells from 100/min to 3054/min (mean 824 ± 693) but remained almost constant for each single neuron during recording periods of 20–30 min The mean amplitude of the currents varied between cells and ranged

from 133 to 2085 pA (mean 707 ± 447 pA). Large amplitude fluctuations also occurred within each recording (see Fig. 1A, top).

3.2. Effect of losigamone on native synaptic currents

Losigamone dose dependently decreased the frequency of the postsynaptic currents (see Fig. 1). The effect occurred rapidly following bath application of the substance and could be reversed completely within 2 min after superfusion of drug-free medium. We tested concentrations between 10 and 100 μM of the drug. The frequency of synaptic currents was significantly lowered at concentrations above 25 μM with a reduction by about half (55 ± 13%) at 75 μM (see Fig. 1B, top). In contrast to the frequency, the mean amplitudes remained largely unaltered with all concentrations of losigamone (see Fig. 1B, bottom). The apparent mean amplitude increase at concentrations higher than 10 μM was not significant. In contrast to the mean amplitude, the cumulative amplitude probability distribution of native currents showed significant changes

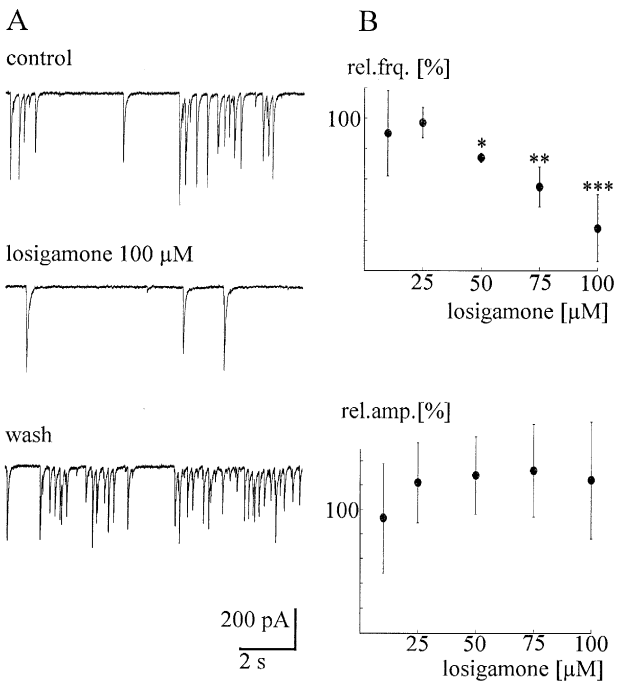

Fig. 1. Modulation of spontaneous synaptic activity by losigamone. (A) Synaptic currents recorded in whole-cell configuration from a cultured rat hippocampal neuron. (Top) Native currents. (Middle) Addition of losigamone (100 μM) reduced the frequency of postsynaptic currents without obvious changes in amplitude or shape. (Bottom) Recovery of the activity 2 min after washout of losigamone. (B) Effects of losigamone on frequency and amplitude of postsynaptic currents. (Top panel) Dose-dependent decrease of current frequency in cultured hippocampal neurons by various concentrations of losigamone. The number of events/min was significantly reduced by 50 μM (to 74 ± 3% of control; $n = 5$), 75 μM (to 55 ± 13%; $n = 7$) and 100 μM losigamone (to 28 ± 22%; $n = 14$). (Bottom panel) The mean amplitude of the events remained unchanged with losigamone. The apparent increase at higher concentrations is not significant. * $P = 0.04$; ** $P = 0.02$; *** $P = 0.001$.

with losigamone in three of five cells analysed ($P < 0.05$). However, in the remaining two cells there was no difference in the distribution between the control and 100 μM losigamone application ($P > 0.4$) although the frequency reduction was present.

The solvent, DMSO, had no effect (see Section 2). The input resistance of the cells was not changed by the substance (control 328 ± 101 MΩ and 336 ± 134 MΩ with losigamone, $P > 0.4$).

A decrease in frequency of spontaneous postsynaptic currents can be mediated by presynaptic inhibition. Therefore, we compared the effect of losigamone with that of the $GABA_B$ receptor agonist, (−)-baclofen (20 μM). This agent reduced the frequency of spontaneous synaptic currents to 66 ± 22% of control values ($n = 8$; $P < 0.02$). However, in contrast to losigamone, baclofen changed the discharge pattern of the synaptic network and resulted in bursts of postsynaptic currents in all cells tested ($n = 8$). Moreover, the mean amplitude showed some tendency to decline (83 ± 5.6% of control) although this effect was not significant ($P = 0.09$).

In order to test whether the effect of losigamone depends on functional Na^+ channels we recorded miniature postsynaptic currents after application of 1 μM tetrodotoxin. The remaining events were smaller and less frequent than native spontaneous PSCs ($n = 7$; mean amplitude 19 ± 6 pA; mean frequency 137 ± 148 events/min; see Fig. 2A, top). Losigamone (100 μM) did not influence the frequency of miniature postsynaptic currents (mPSCs; 177 ± 154 events/min; $n = 7$; $P = 0.50$; see Fig. 2A, bottom) and also left the mean amplitude constant (19 ± 5 pA, see Fig. 2B). The cumulative amplitude probability of events was compared in four cells and did not show any significant differences (see Fig. 2C; $P > 0.25$, Kolmogorov-Smirnov test). In one cell we fitted a monoexponential curve to the decaying part of averaged currents which showed a similar time constant under control conditions and with losigamone (3.4 vs. 3.2 ms, respectively). Thus, the substance does not influence miniature synaptic currents recorded in the presence of tetrodotoxin.

3.3. Effect of losigamone on IPSCs and EPSCs

Inhibitory postsynaptic currents were isolated by addition of the glutamate receptor blockers NBQX (10 μM) and (±)-APV ((±)-2-amino-5-phosphonopentanoic acid; 30 μM). With these substances, the frequency of spontaneous postsynaptic events decreased to 27 ± 37% of the control values ($n = 19$). The remaining currents were completely blocked by 20 μM bicuculline, indicating that they were $GABA_A$ receptor-mediated IPSCs. In order to enhance the frequency of IPSCs sufficiently for analysis, we added 1 mM 4-aminopyridine (Rutecki et al., 1987; Traub et al., 1995). IPSCs were then sampled from six cells. The currents decayed bi-exponentially with $\tau_{fast} = 7.0 \pm 4.2$ ms

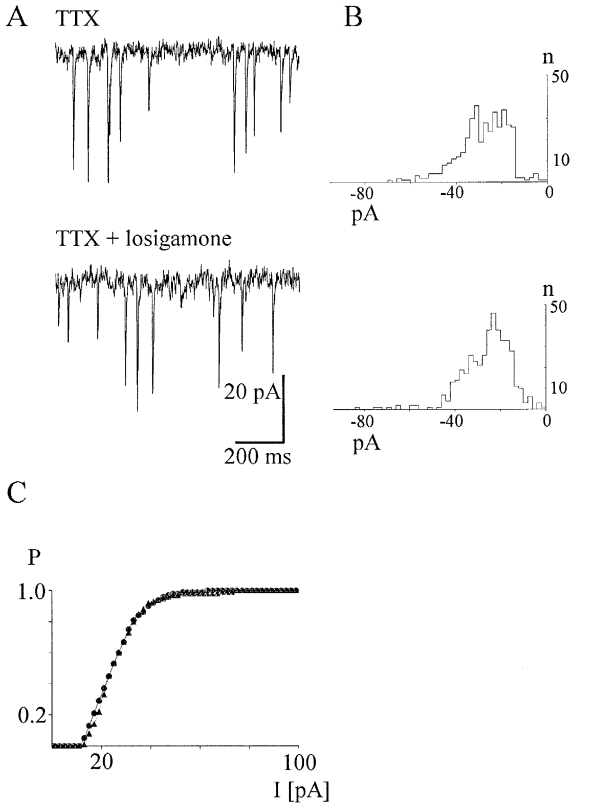

Fig. 2. Losigamone did not affect miniature postsynaptic currents. (A) (Top) Spontaneous synaptic activity recorded in whole-cell configuration with 1 μM tetrodotoxin. Current amplitudes were generally small. (Bottom) Losigamone (100 μM) did not alter the frequency of the events. (B) Amplitude histograms were largely similar without and with losigamone. Data from 1 min of recordings from the same cell (total of 395 events with tetrodotoxin and 434 events with tetrodotoxin + losigamone). (C) Cumulative probability of amplitude without (circles) and with (triangles) losigamone reveals no difference in amplitude distribution (Kolmogorov-Smirnov test: $P > 0.7$).

and $\tau_{slow} = 29.5 \pm 9.8$ ms. The fast component contributed $30 \pm 10\%$ to the total amplitude. Losigamone (100 μM) reduced the frequency of the spontaneous IPSCs to $13 \pm 12\%$ of the control values ($P < 0.02$). The mean amplitude and both decay time constants remained unchanged ($P > 0.10$; Wilcoxon test).

Conversely, we tried to isolate excitatory postsynaptic currents. Upon addition of bicuculline (20 μM) and picrotoxin (40 μM) the postsynaptic currents vanished initially. However, after 1–2 min we observed a new pattern of burst-like activity (see Fig. 3, top). Losigamone (100 μM) reduced the frequency of single synaptic currents from 89 ± 43 per min to 28 ± 22 ($n = 4$ cells), mainly due to a reduction in the number of single events within each burst (8 ± 6 under control conditions vs. 3 ± 2 under losigamone; see Fig. 3, bottom).

3.4. Effect on evoked IPSCs

Evoked IPSCs were recorded in the presence of NBQX and (±)-APV by local extracellular electrical stimulation

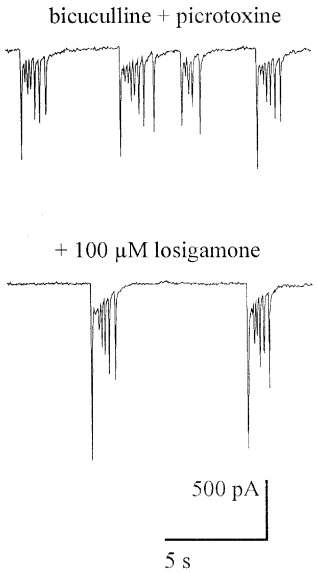

Fig. 3. Effect of losigamone on isolated EPSCs. (Top) Addition of 20 μM bicuculline and 40 μM picrotoxin resulted in bursts of synaptic currents. (Bottom) Losigamone (100 μM) reduced the frequency of bursts and the number of single synaptic events within each burst.

of neighboring cells with a second patch pipette. At stimulation intensities between 9 and 42 V (0.2 ms) we could reliably elicit synaptic currents (see Fig. 4, top). The averaged IPSC amplitude varied between cells from 66 to 590 pA ($n = 5$ cells). The IPSCs followed a bi-exponential time-course with $\tau_{fast} = 10.3 \pm 3.6$ and $\tau_{slow} = 48.2 \pm 21.2$ where the fast component covered $55 \pm 11\%$ of the total

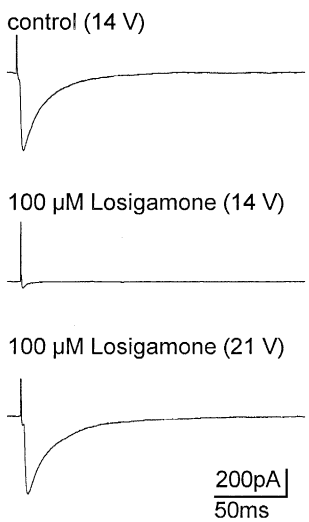

Fig. 4. Effect of losigamone on evoked IPSCs. (Top) GABAergic IPSCs were recorded after addition of 10 μM NBQX and 30 μM (±)-APV by electrical stimulation of a neighboring neuron with a second patch pipette. Average of 20 single IPSCs. The current decay is bi-exponential (τ_{fast} = 10.9 ms; τ_{slow} = 38.6 ms; amplitude 590 pA). (Middle) Upon addition of losigamone the same stimulation did not elicit synaptic currents. (Bottom) Recovery of the currents by increased stimulation strength in the presence of 100 μM losigamone. Time course and amplitude were largely unchanged from the control value (τ_{fast} = 10.1 ms; τ_{slow} = 32.7 ms; amplitude 501 pA).

amplitude. In all five cells analysed, the addition of losigamone (100 μM) reversibly abolished the evoked IPSCs (see Fig. 4, middle). However, in three neurons the currents could be restored by increases of the stimulation voltage to 150–190% of the control values (see Fig. 4, bottom). Thereafter, the GABAergic currents appeared unaltered as compared to the control (amplitude 88 ± 43% of control; τ_{fast} 11.3 ± 2.4 ms; τ_{slow} 58.4 ± 24.7 ms).

3.5. Effect on action potential frequency

Thus, losigamone lowers the excitability of neurons and the frequency of spontaneous synaptic activity. In order to monitor the spontaneous discharge behaviour of the neurons without perfusing the cell with the artificial pipette solution, we recorded from cells in the cell-attached configuration. Action potentials could be clearly distinguished as fast, biphasic current or voltage deflections in voltage or current clamp mode, respectively (see Fig. 5A, top). In all cells analysed, 100 μM losigamone decreased the frequency of these events from an average of 133 ± 168 per min to 18 ± 20% of control ($n = 7$; $P < 0.02$; see Fig. 5A (middle) and 5B). The effect was reversible in all cells upon washout of the substance.

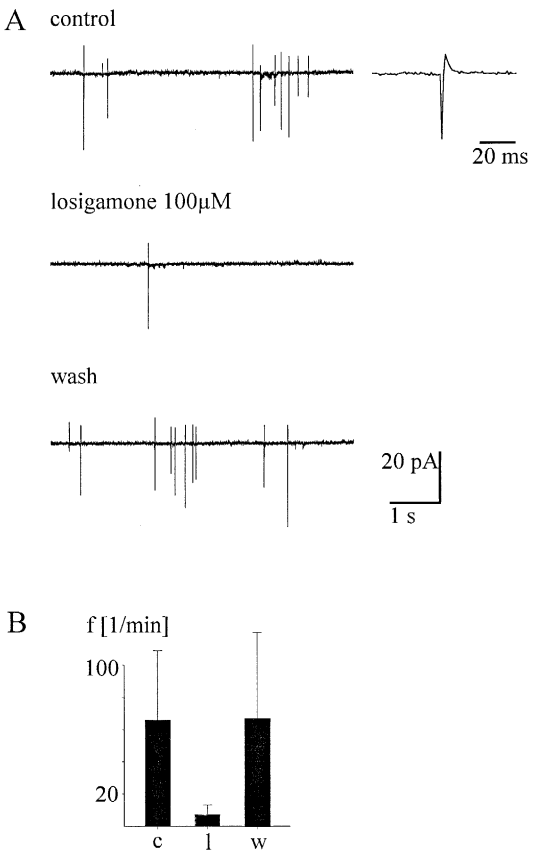

Fig. 5. Effect of losigamone on action potentials. (A) (Top) Cell-attached voltage-clamp recording from a cultured neuron revealing spontaneous activity. Expanded trace shows the stereotypic fast events corresponding to action potentials. (Middle) Losigamone reduced the frequency of the events. (Bottom) Recovery upon washout of losigamone for 2 min. (B) Histogram showing the data from 7 cells. Frequency (miniature currents per minute) was reversibly decreased with losigamone ($P < 0.02$).

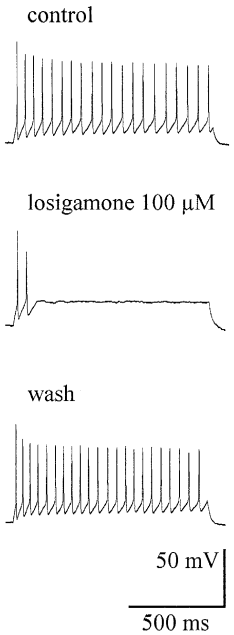

Fig. 6. Suppression of repetitive action potential firing by losigamone. (Top) Injection of depolarizing current (70 pA) yielded repetitive discharges in a neuron in whole-cell current clamp recording. (Middle) Losigamone abolished the repetitive action potentials without visible change in input resistance. (Bottom) The effect was fully reversible upon washout.

We also recorded five cells in the current-clamp mode of the whole-cell configuration and elicited action potentials by current injection pulses of 1 s duration (resting potential −60 to −65 mV; amplitude 30–70 pA). Whereas all cells showed repetitive action potentials under control conditions, losigamone reversibly abolished this behaviour and resulted in flat depolarizations after one or two action potentials (see Fig. 6).

4. Discussion

Our data indicate that losigamone generally reduces the frequency of synaptic events in a network of cultured hippocampal neurons through a presynaptic mechanism. The findings are consistent with previous observations made in rat brain slices. Recordings from CA1 and entorhinal cortex neurons showed that the substance reduces the intrinsic excitability of neurons with only minor effects on postsynaptic potentials (Schmitz et al., 1995). These intracellular recordings revealed the same suppression of repetitive discharge behaviour as observed in our patch clamp experiments with dialysed cells. Moreover, the substance has been successfully applied to the low-Ca^{2+} epilepsy model in vitro (Köhr and Heinemann, 1990b), which is independent from synaptic transmission (Konnerth et al., 1986; Jefferys and Haas, 1982; Jefferys, 1995).

This effect also emphasises the importance of intrinsic neuronal properties for the mechanism of action of losigamone. Together, previous data and the present results make it likely that the main anticonvulsive effect of losigamone is due to the reduction of intrinsic excitability of neurons.

In contrast to these findings, it has recently been suggested that losigamone enhances the GABA-induced Cl^- influx in cultured spinal cord neurons (Dimpfel et al., 1995), which might indicate postsynaptic strengthening of GABAergic inhibition. We therefore looked specifically for any modulation of GABA-mediated IPSCs by losigamone. Anticonvulsive $GABA_A$ receptor modulators such as the benzodiazepines and barbiturates result in prolonged or enhanced IPSCs (Waterhouse and DeLorenzo, 1996). Other antiepileptic modulators of the GABAergic system yield an enhanced inhibition by blocking GABA uptake, which will also cause prolonged IPSCs (Suzdak and Jansen, 1995; Heit and Schwark, 1988; Smith et al., 1995; Pfeiffer et al., 1996). The addition of 4-aminopyridine, which was necessary to enhance synaptic activity sufficiently for analysis, might have influenced the presynaptic discharge mode (Traub et al., 1995). However, any postsynaptic modulation of the $GABA_A$ receptors would still result in an alteration of amplitude or time course of the postsynaptic signal. Our findings make it very unlikely that losigamone affects GABA-mediated inhibition by a postsynaptic mechanism. Losigamone altered neither the time course nor the mean amplitude of spontaneous IPSCs. Evoked IPSCs were primarily abolished by the substance but could be restored after the stimulation intensity was increased. Thereafter, the events were not different from control signals which would be expected from any modulator of the $GABA_A$ receptor (Mody et al., 1994; Otis and Mody, 1992; Roepstorff and Lambert, 1994). Losigamone also depressed the frequency of glutamatergic excitatory postsynaptic currents (EPSCs) recorded during $GABA_A$ receptor blockade. It is therefore not likely that the main mechanism of action of losigamone involves a specific block of postsynaptic neurotransmitter receptors.

Thus, our findings support a presynaptic reduction of excitability by losigamone. A common presynaptic modulatory mechanism in hippocampal neurons is the G-protein-mediated inhibition of transmitter release activated by $GABA_B$ receptors (Thompson et al., 1993). In our experiments, (−)-baclofen switched the synaptic activity into a rhythmic pattern which we never observed under losigamone. Moreover, this substance reduces the amplitude of GABAergic IPSCs in cultured hippocampal neurons (Harrison, 1990) and in brain slices (Blaxter and Carlen, 1985; Misgeld et al., 1986; Thompson and Gähwiler, 1992; Morishita and Sastry, 1994), an effect which we did not observe with losigamone. Thus, our data do not favour a G-protein-mediated modulation of transmitter release by losigamone.

The fact that the frequency of EPSCs was reduced in the same manner as the frequency of IPSCs raises the question of how the net anticonvulsive effect of the substance can be explained. However, hippocampal neurons receive a steady inhibitory input of action potential-independent miniature IPSPs located close to the soma (Soltesz et al., 1995). Losigamone has no effect on these signals and therefore, one important source of neuronal inhibition is not affected by losigamone. Moreover, network models and experimental work have shown that synchronous discharges can result from increased excitability of neurons, even with intact inhibition, and that selective disinhibition can reduce instead of enhance this activity under certain circumstances (Traub et al., 1995). Chronic epileptic syndromes can even co-exist with increased GABAergic inhibitory transmission (Whittington and Jefferys, 1994). Therefore, anticonvulsive drugs do not necessarily act by enhancing synaptic inhibition but may also exert their network effect by generally reducing neuronal excitability (Waterhouse and DeLorenzo, 1996; Coulter et al., 1990).

Anticonvulsants such as phenytoin, carbamazepine and valproic acid reduce excitability through effects on Na^+ channels (Waterhouse and DeLorenzo, 1996). Such a mechanism might indeed underlie the failure of repetitive action potential firing under losigamone as observed in our experiments and by Schmitz et al. (1995). Therefore, we recorded miniature postsynaptic currents in the presence of tetrodotoxin. Losigamone did not affect the frequency, mean amplitude, cumulative amplitude distribution and decay time course of these signals. Thus, functional Na^+ channels are necessary for elicitation of the effect of the substance. It remains to be found whether the mechanism of action is similar to that of phenytoin, which alters the kinetic properties of voltage-dependent Na^+ channels (Chao and Alzheimer, 1995).

In summary, our data show a general decrease in neuronal discharge frequency and synaptic excitability caused by losigamone and support the view that the anticonvulsive effects of the substance might be related to presynaptic and intrinsic neuronal properties rather than to modulation of postsynaptic receptors.

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