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Presynaptic Inhibition of Excitatory Neurotransmission by Lamotrigine in the Rat Amygdalar Neurons

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KEY WORDS Lamotrigine, Anticonvulsant, Calcium channels, Amygdala, Synaptic transmission

ABSTRACT Lamotrigine (LAG) is a new antiepileptic drug which is licensed as adjunctive therapy for partial and secondary generalized seizures. In the present study, the mechanisms responsible for its antiepileptic effect were studied in rat amygdaloid slices using intracellular recording and whole-cell patch clamp techniques. Bath application of LAG (50 μM) reversibly suppressed the excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) evoked by stimulating ventral endopyriform nucleus. Synaptic response mediated by the N-methyl-D-aspartate (NMDA) receptor ($EPSP_{NMDA}$) was isolated pharmacologically by application of a solution containing non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX,10 μM) and γ-aminobutyric $acid_A$ receptor antagonist bicuculline (20 μM). LAG produced a parallel inhibition of $EPSP_{NMDA}$. Postsynaptic depolarization induced by α-amino-5-methyl-4-isoxazole propionate (AMPA) was not altered by LAG. In addition, LAG increased the ratio of the second pulse response to the first pulse response (P_2/P_1), which is consistent with a presynaptic mode of action.

The L-type Ca^{++} channel blocker nifedipine (20 μM) had no effect on LAG-induced presynaptic inhibition. However, the depressant effect of LAG was markedly reduced in slices pretreated with N-type Ca^{++} channel blocker ω-conotoxin-GVIA (ω-CgTX-GVIA, 1 μM) or a broad spectrum Ca^{++} channel blocker ω-conotoxin-MVIIC (ω-CgTX-MVIIC, 1 μM). It is concluded that a reduction in ω-CgTX-GVIA-sensitive Ca^{++} currents largely contributes to LAG-induced presynaptic inhibition.

INTRODUCTION

Lamotrigine (LAG) is a new anticonvulsant of therapeutic potential for the treatment of partial and secondarily generalized seizures (Macdonald and Kelly, 1995; Messenheimer, 1995; Upton, 1994). Previous studies of animal seizure models have shown that LAG is able to block maximal electroshock (MES) and pentylenetetrazole-induced hindlimb extension (Miller et al., 1986), a characteristic shared by the currently used antiepileptic agents against generalized tonic-clonic and partial seizures. LAG also reduced, but not blocked, the duration of kindling-induced afterdischarges (O'Donnell and Miller, 1991).

The antiepileptic effect of LAG is usually attributed to presynaptic inhibition of excitatory neurotransmitter release. LAG is effective in inhibiting the release of glutamate and aspartate evoked by the Na^+ channel activator veratrine (Leach et al., 1986). At therapeutically relevant concentrations, LAG blocked high-frequency sustained firing of Na^+-dependent action potentials in cultured mouse spinal cord neurons and displaced [^{3}H]batrachotoxin from its Na^+ channel binding site in brain synaptosomes (Cheung et al., 1992). It is therefore proposed that LAG acts by blocking voltage-dependant Na^+ channels, thus stabilizing presynaptic neuronal membrane and reducing the release of transmitter. However, the question whether LAG-induced inhibition of transmitter release is due to its specific interaction with Na^+ channel remained to be determined.

In this study, we examined the effects of LAG on the synaptic transmission and the epileptiform activity induced by bicuculline. By the use of selective antago-

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nists for Ca^{++} channels, we have demonstrated that N-type but not L-type channels were likely involved in LAG modulation of neurotransmitter release at rat amygdalar excitatory synapses. The basolateral amygdala was selected for study because experimental and clinical evidence indicated that the amygdala is important in the initiation and spread of seizures (Ben-Ari et al., 1980; Racine and Burnham, 1984).

MATERIALS AND METHODS

Preparation of the Amygdalar Slice

The procedures for the preparation of the amygdalar slices were similar to that reported previously (Gean and Chang, 1992). In brief, male Sprague-Dawley rats weighing 125-200 grams were decapitated and the brains rapidly removed from the skull. Coronal slices nominally 500 μm thick were cut and the appropriate slices were placed in a beaker of artificial cerebral spinal fluid (ACSF). The ACSF was bubbled continuously with 95% O_2-5% CO_2 to maintain the proper pH (7.3–7.5). The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, and glucose 11.

A single slice was transferred to the recording chamber where it was held submerged between two nylon nets and maintained at 32 ± 1°C. The chamber consisted of a circular well of a low volume (1–1.5 ml) and was constantly perfused at a rate of 2–3 ml/min. A bipolar stimulating electrode (SNE-200X, Kopf Instruments, Tujunga, CA) was placed in the ventral endopyriform nucleus, close to the recording electrode (1–2.5 mm apart). Orthodromic stimuli were delivered with monophasic constant voltage pulses from a Grass S88 stimulator with isolation unit. Stimulus intensities varied between 5 and 50 V with a pulse duration of 50 μs. For most experiments, stimulus intensities were adjusted to just subthreshold for orthodromic spike generation.

Recording Methods

Intracellular recordings were obtained from neurons of the basolateral amygdaloid (BLA) nucleus using conventional intracellular techniques. Microelectrodes were pulled from microfiber-filled 1.0 mm capillary tubing on a Brown-Flaming electrode puller (Sutter Instruments, Novato, CA). The electrodes were filled with 3 M KCl with resistance ranging from 60–120 MΩ. Membrane input resistance was measured by passing a current of 50 ms duration and recording the resultant electrotonic potentials. Electrical signals were amplified using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) and recorded on a Gould 3200 chart recorder. Very fast transient potentials, which could not be adequately resolved by the chart recorder, were digitized using a Data-6100 digital oscilloscope (Data Precision Co., Peabody, MA) and were reproduced on a Hewlett-Packard plotter.

Excitatory postsynaptic currents (EPSCs) were recorded from basolateral amygdala neurons that were obtained using previously described "blind" whole-cell recording techniques (Blanton et al., 1989). The tips of whole-cell pipettes were filled with a solution containing 115 mM cesium gluconate, 5 mM NaCl, 1 mM EGTA, 0.3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaATP, 0.4 mM NaGTP, and 10 mM HEPES, adjusted to pH 7.3 with KOH and 280 mOsm with sucrose. For data acquisition and analysis, pClamp 6.0 (Axon Instruments) running on PC486 was used. All data were expressed as mean ± SEM. Statistical analysis was performed using the Student's t-test and a P-value of less than 0.05 was considered to be statistically significant.

Drug Application

Lamotrigine, nifedipine, and CNQX were dissolved in a dimethylsulfoxide (DMSO) stock solution and kept frozen until the day of experiment. It was then added to the ACSF to adjust the final concentration. The concentrations of DMSO in the perfusing medium ranged from 0.1 to 0.25% which had no effect on the synaptic responses or the passive properties of the BLA neurons. Changes in the superfusing solution were done by switching a 3-way stopcock to an alternative reservoir. Lamotrigine was kindly supplied by the Wellcome Foundation Ltd. Nifedipine and bicuculline were obtained from Sigma Chemicals (St. Louis, MO); ω-conotoxin-GVIA, ω- conotoxin-MVIIC, and CNQX from Research Biochemicals International (Natick, MA).

RESULTS

Antiepileptic Effect of LAG

In the presence of the $GABA_A$ receptor antagonist, evoked and spontaneous epileptiform discharges could be recorded in the basolateral amygdala neurons, as described previously (Gean and Shinnick-Gallagher, 1987). Consistent with previous reports in cultured neurons (Lees and Leach, 1993), bath application of LAG shortened the duration of burst discharges. As shown in Figure 1A, 2 min after introduction of LAG (50 μM) into the perfusion chamber, a reduction of burst duration was observed. With continued perfusion, the burst duration was decreased by an average of 50.8% (control: 358 ± 100 ms; LAG: 176 ± 38 ms; wash: 374 ± 121 ms; $n = 8$; $P < 0.02$). The effect of LAG was reversible within 15 min of washing. A transient hyperpolarizing current pulse (0.2 nA, 50 ms) was applied before the evoked responses to monitor the neuronal input resistance. As noted in this neuron, LAG had no effect on the input resistance.

LAG Produced Synaptic Depression

In the amygdala, afferent stimulation evoked an excitatory postsynaptic potential (EPSP) that is mediated by excitatory amino acids acting predominantly on the AMPA receptors with a small contribution from NMDA receptors (Gean and Chang, 1992). Superfusion of LAG

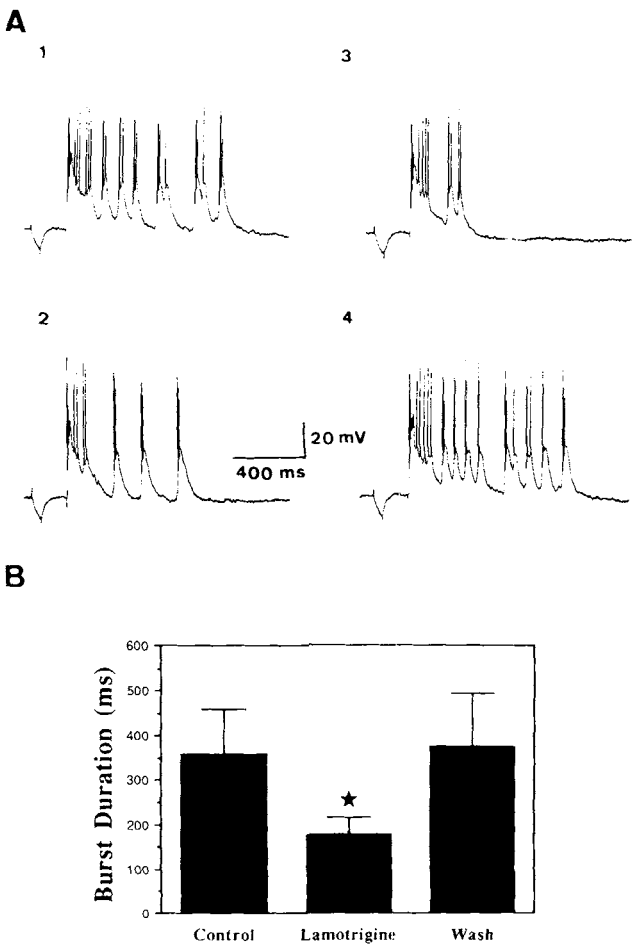


Fig. 1. Effect of LAG on the epileptiform activity induced by bicuculline. A_1: In the presence of bicuculline (20 μM), afferent stimulation evoked a burst response. A_2,A_3: Two and 10 min after application of LAG (50 μM). A_4: Response 15 min after beginning washout of LAG. A hyperpolarizing current pulse (0.2 nA, 50 ms) was passed through the recording electrode to monitor input resistance. **B:** Graphic analysis of the effect of LAG on the epileptiform activity. Bar graphs represent the mean ± SE of the bursting duration. Duration of burst responses was measured as the time from the initial depolarization to 90% of its decay phase. $^*P < 0.02$ vs. control.

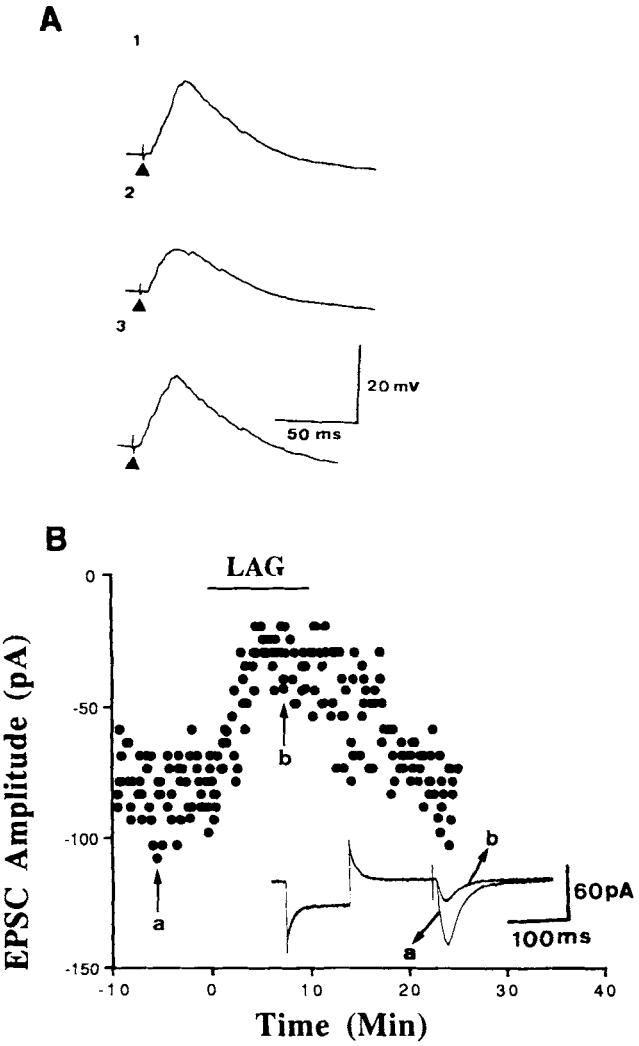
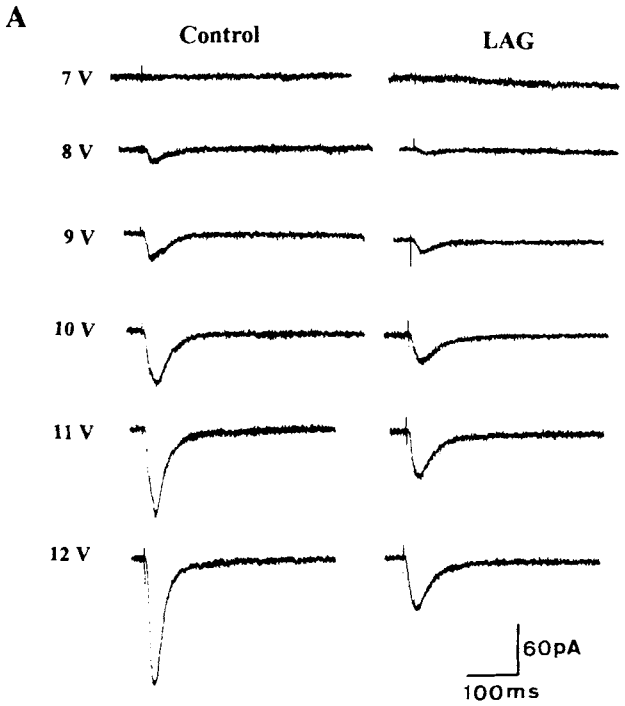


Fig. 2. Effect of LAG on the normal synaptic transmission. A_1: Control EPSP prior to LAG application. A_2,A_3: EPSPs recorded from the same cell during application of 50 μM LAG (A_2) and 15 min after beginning washout of LAG (A_3). **B:** Time course of the effect of LAG on EPSC amplitude in one representative cell. **Inset:** The superimposed traces of 10 averaged responses taken before and during application of LAG (50 μM). A hyperpolarizing voltage step (5 mV, 200 ms) was passed through the pipette electrode to monitor input resistance.

consistently suppressed the EPSP. As shown in Figure 2A, application of LAG (50 μM) for 10 min reduced the amplitude of EPSP. At concentrations of 10 and 50 μM, LAG suppressed the amplitude of EPSP by an average of 52.0 ± 6.8% (n = 7) and 63.2 ± 7.6% (n = 14) respectively. There was no significant difference in LAG effect between 10 and 50 μM, suggesting that the response may be saturated by 10 μM. The effect of LAG was readily reversible when slices were superfused with control ACSF that did not contain the drug. We also tested the effect of LAG using whole-cell recordings. In all neurons, LAG affected neither holding current nor input resistance. In agreement with the intracellular data, LAG (50 μM) similarly suppressed the amplitude of EPSCs by 42.9 ± 4.6% (n = 10, $P < 0.01$) (Fig. 2B). This inhibitory action of LAG on the EPSCs was reversible on washing with control solution for 10 to 20 min. We analyzed the input-output relationship of the EPSCs because LAG could reduce action potential firing of presynaptic fibers in a manner that is overcome by higher stimulus intensity. Figure 3 shows the amplitude of EPSCs as a function of stimulus intensity. LAG reduced the amplitude of EPSCs at all intensities tested.

We next tested the effect of LAG on the NMDA receptor-mediated EPSP ($EPSP_{NMDA}$) because if LAG acts on presynaptic site to reduce glutamate release, then LAG should produce a parallel decrease in the $EPSP_{NMDA}$. In the presence of AMPA receptor antagonist CNQX (10 μM), and $GABA_A$ receptor antagonist bicuculline (20 μM), a depolarizing potential persisted that could be



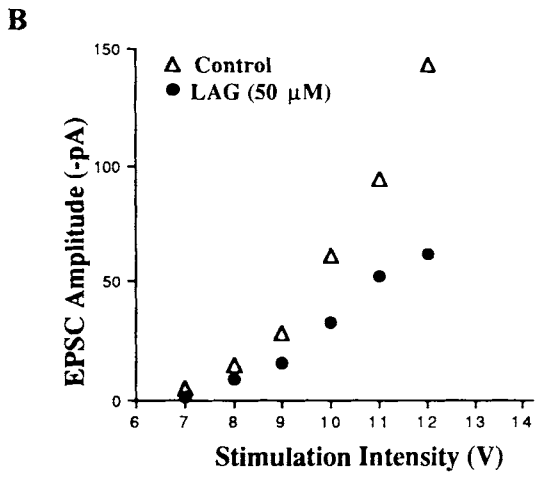


Fig. 3. Effect of LAG on the input-output relationship for EPSCs. **A:** The amplitude of EPSC was reduced by LAG (50 μM) at various stimulus intensities in a typical cell. **B:** Plot of the input-output relationship obtained in A. The cell was held at −70 mV.

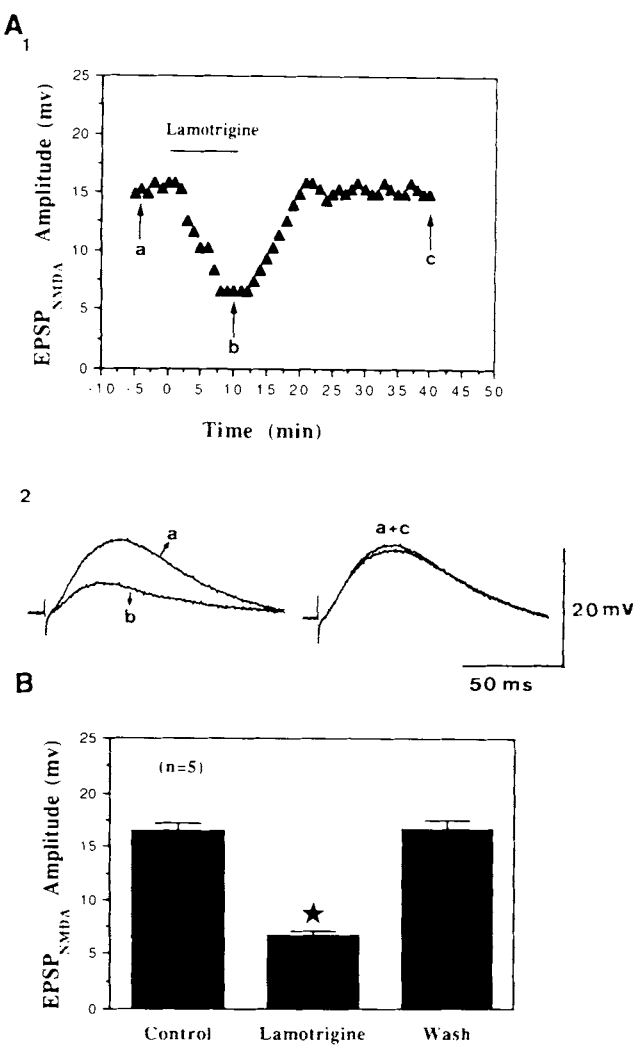


Fig. 4. Inhibition of $EPSP_{NMDA}$ by LAG A_1: The $EPSP_{NMDA}$ amplitude was plotted against time. Bar denotes the period of delivery of 50 μM LAG. A_2: Superimposed traces taken at different times indicated in A_1. Experiments were performed in the presence of CNQX (10 μM) and bicuculline (20 μM). **B:** Graphic analysis of the effect of LAG (50 μM) on the $EPSP_{NMDA}$. $^*P < 0.001$.

abolished by D-APV (20 μM). This APV-sensitive depolarizing potential was therefore considered to be mediated by activation of NMDA receptors (Gean et al., 1992). Application of LAG (50 μM) reversibly reduced the amplitude of $EPSP_{NMDA}$ by an average of 59.4% in all five neurons tested (Fig. 4).

LAG Does Not Affect Postsynaptic Responses to AMPA

LAG had no measurable effect on either the resting membrane potential or the input resistance. Resting membrane potential differed by <2 mV relative to baseline values and no clear pattern of depolarization or hyperpolarization was observed during application of LAG. Input resistance was 47.6 ± 3.6 MΩ before and 46.6 ± 2.8 MΩ (n = 7, $P > 0.1$) during LAG treatment.

We examined the effect of LAG on the postsynaptic responses to exogenously applied AMPA. The experiments were performed in the presence of tetrodotoxin (0.5 μM). As illustrated in Figure 5A, superfusion of AMPA produced a membrane depolarization (30.1 ± 6.4 mV, n = 10). LAG (50 μM) pretreatment did not affect the AMPA-induced membrane depolarization (31.4 ± 6.5 mV, n = 10, $P = 0.51$) (Fig. 5B).

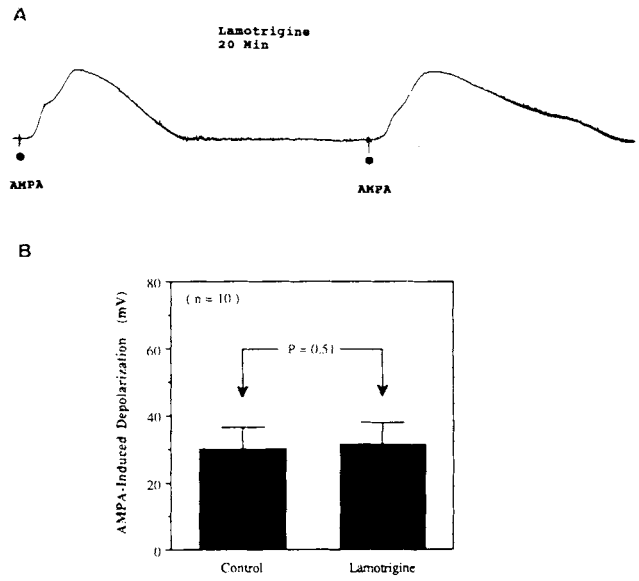


Fig. 5. Effect of LAG on AMPA- induced membrane responses. Superfusion of AMPA (10 μM, 1 min) evoked a membrane depolarization. **A:** Pretreatment of the slice with LAG (50 μM) for 20 min did not significantly affect AMPA-induced depolarization. **B:** Graphic analysis of the effect of LAG pretreatment on the AMPA-induced membrane depolarization.

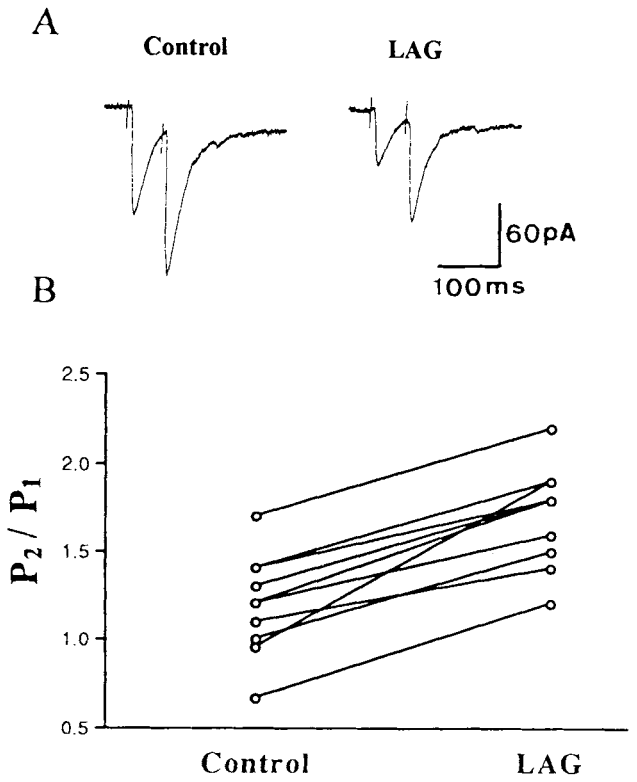


Fig. 6. The effect of LAG on paired-pulse modification of EPSCs. **A:** Sample records of EPSCs evoked by paired stimuli (60 ms interval) in control condition and after application of LAG (50 μM). **B:** Data from 10 cells showing effect of 50 μM LAG on paired pulse modification of EPSCs.

LAG Changes Paired-Pulse Modulation of EPSCs

Paired-pulse modification of neurotransmission has been studied extensively and is attributed to a presynaptic change in release probability (Manabe et al., 1993; Schulz et al., 1994). An increase in the ratio of the second pulse response to the first pulse response (P_2/P_1) indicates a decrease in release probability, because manipulations that depress transmitter release usually increase the magnitude of P_2/P_1. Therefore, we compared the magnitude of P_2/P_1 before and after the treatment with LAG. Synaptic responses to a pair of stimuli were recorded with interstimulus interval of 60 ms. Figure 6 is a summary of 10 experiments showing that LAG increased the magnitude of P_2/P_1 in all neurons tested. In control solution, P_2/P_1 was 1.2 ± 0.1. Addition of 50 μM LAG to the superfusion solution increased P_2/P_1 to 1.7 ± 0.1 ($P < 0.001$, paired t-test, n = 10).

Involvement of Ca^{++} Channels in LAG-Induced Synaptic Depression

The release of neurotransmitter is largely dependent on the activation of voltage-dependent Ca^{++} channels (VDCCs) present in the nerve terminals (Llinas et al., 1981;Wheeler et al., 1994). We investigated the involvement of specific Ca^{++} channel subtypes in the LAG-induced synaptic depression with the use of selective blockers. Nifedipine, a selective L-type Ca^{++} channel blocker, affect neither the EPSP amplitude nor the LAG-induced synaptic depression. In the presence of nifedipine (20 μM), LAG (50 μM) reduced the EPSP amplitude by an average of 62.6 ± 3.9% (n = 5) (Fig. 7), which was not significantly different from the inhibition produced by LAG alone (63.2 ± 7.6%, n = 14, $P > 0.1$, unpaired t-test).

Application of N-type Ca^{++} channnel blocker, ω-conotoxin-GVIA (ω-CgTX-GVIA) (Aosaki and Kasai, 1989; Fox et al., 1987), at a saturating concentration of 1 μM reduced the amplitude of EPSP by 89.1 ± 4.1% (n = 7). Because the inhibition was large, we usually had to increase the stimulus intensity to restore the EPSP amplitude in order to facilitate the comparison of the effect of LAG before and after toxin application. In Figure 8A, application of ω-CgTX-GVIA (1 μM) depressed the EPSP. After the effect of toxin had stabilized, EPSP amplitude was increased by increasing the stimulus intensity to near control level. Addition of LAG (50 μM) under this condition reduced the EPSP amplitude only by 6.6 ± 2.5% (n = 7) which was significantly less than when LAG was applied alone (Fig. 8B).

Similar experiments were performed with the use of a broad spectrum Ca^{++} channel blocker, ω-conotoxin-MVIIC (ω-CgTX-MVIIC), which affects N-, P-, and Q-type channels (Hillyard et al., 1992; Wheeler et al., 1994; Wu and Saggau, 1995; Zhang et al., 1993). As

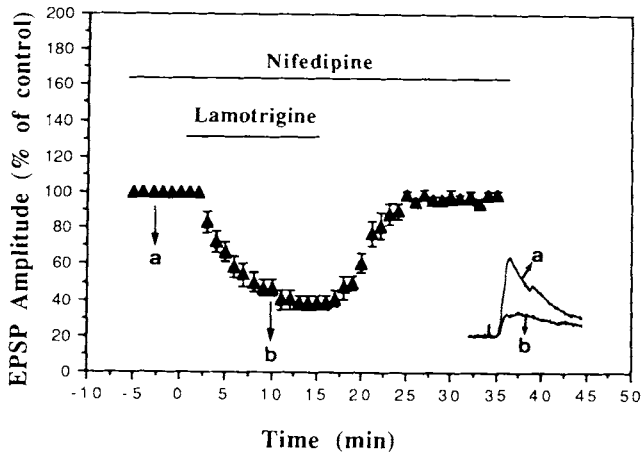


Fig. 7. Pretreatment with nifedipine did not affect the LAG-induced synaptic depression. The percent changes of EPSP amplitude were plotted against time. Bars denote the periods of delivery of 20 μM nifedipine and 50 μM LAG. **Inset:** The superimposed traces taken at different times as indicated.

illustrated in Figure 9A, ω-CgTX-MVIIC (1 μM) reduced the EPSP amplitude by 71.6 ± 6.3% (n = 8). Raising the stimulus intensity restored the amplitude to near pre-toxin level. Under this condition, LAG reduced excitatory transmission by an average of 11.0 ± 5.1% which was significantly less than inhibition produced in the absence of toxin (Fig. 9B).

DISCUSSION

LAG-Induced Synaptic Depression

The results obtained in the present study indicate that LAG inhibits synaptic transmission primarily by a presynaptic mechanism. First, LAG suppressed the EPSPs and EPSCs with negligible changes in resting membrane potential and input resistance of postsynaptic neurons and the depression is accompanied by an increase in the ratio of the second pulse response to the first pulse response (P_2/P_1). Second, postsynaptic responses to excitatory amino acid receptor agonist was not significantly altered by LAG. Third, both non-NMDA and NMDA receptors-mediated EPSPs were depressed to approximately the same degree upon perfusion of LAG. A decrease in transmitter release readily explained a parallel inhibition of both components of the evoked EPSPs. Finally, the effect of LAG was occluded by a specific type of Ca^{++} channel blockers. Previous biochemical studies revealed that LAG produced a dose-related protection against kainate-induced neurotoxicity but had no effect on ibotenic lesion in the striatum (McGeer and Zhu, 1990). Neurotoxicity caused by kainate depended on the release of neuronal glutamate, whereas that produced by ibotenic acid was independent of glutamate. These studies lent support to our conclusion that LAG depresses synaptic transmission by inhibiting glutamate release, rather than reducing postsynaptic excitability.

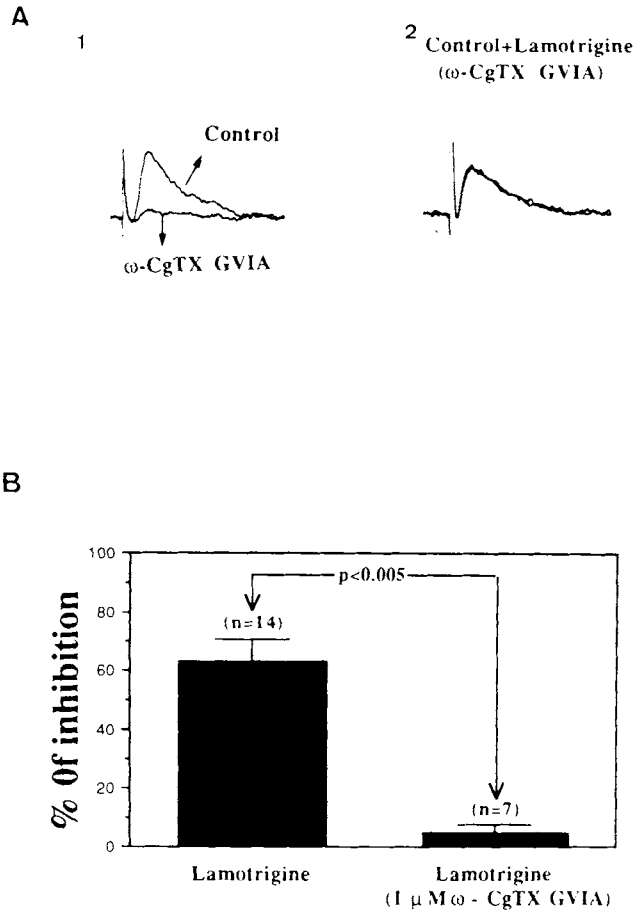


Fig. 8. Pretreatment with ω-CgTX-GVIA markedly reduced LAG-induced synaptic depression. **A:** A typical experiment showing the effects of ω-CgTX-GVIA (1 μM) on the EPSP (A_1) and LAG-induced synaptic depression (A_2). In this cell, ω-CgTX-GVIA reduced the EPSP amplitude to 12.5% of its baseline. The amplitude was restored to near its control level by increasing the stimulus intensity and LAG (50 μM) was then applied. Under this condition, LAG failed to affect the EPSP. **B:** Comparison of the effect of LAG (50 μM) in the absence and in the presence of ω-CgTX-GVIA (1 μM) on the EPSP.

ω-CgTX-GVIA Occluded the Effect of LAG

The precise mechanism through which LAG inhibited synaptic transmission was not conclusively defined. The sensitivity of LAG- induced synaptic depression to subtype-specific Ca^{++} channel blockers provides good evidence that Ca^{++} currents are involved in LAG's action. The use of a broad spectrum Ca^{++} channel blocker ω-CgTX-GVIA to occlude the effect of LAG further strengthens the hypothesis that LAG depresses synaptic transmission by reducing presynaptic Ca^{++} influx. LAG-induced depression of EPSP was unchanged with or without nifedipine preapplication indicating that L-type Ca^{++}channels were not required for the action of LAG. By contrast, when N-type Ca^{++} channels were blocked by a saturating concentration (1 μM) of ω-CgTX-GVIA, the depressant effect of Lag was markedly reduced compared with that without ω-CgTX-GVIA pre-

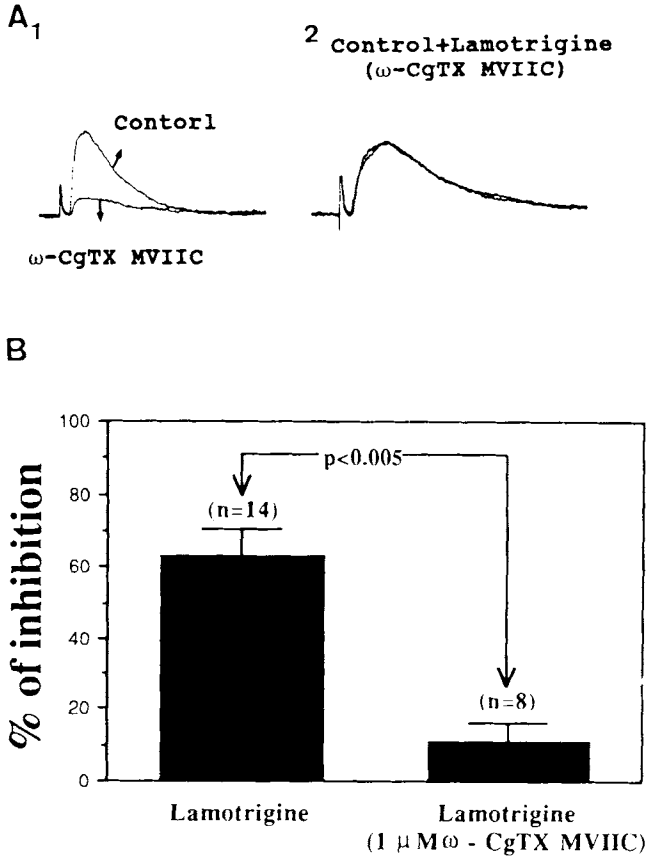


Fig. 9. Reduction of the effect of LAG by ω-CgTX-MVIIC. **A:** A typical experiment showing the effects of ω-CgTX-MVIIC (1 μM) on the EPSP (A_1) and LAG-induced synaptic depression (A_2). **B:** Comparison of the effect of LAG (50 μM) in the absence and in the presence of ω-CgTX- MVIIC (1 μM) on the EPSP.

application. It is suggested that the effect of LAG is mediated largely by modulation of N-type channels. Since LAG still reduced a small portion of EPSP remaining in the presence of ω-CgTX-GVIA, and an exponential relationship between Ca^{++} influx and neurotransmitter release has been demonstrated in the CNS (Wu and Saggau, 1994), it is likely that LAG also inhibits Q- and/or P-type channels. However, the possibility that LAG acted by blocking Na^+ channels, thereby inhibiting glutamate release could not be ruled out, although in the present study the inhibitory effect of LAG on EPSCs was not overcome by an increase in the stimulus intensity.

Therapeutic Implication

LAG has been used for the adjunctive treatment of human partial and generalized tonic-clonic seizures. Mechanistically, drugs that are effective against generalized tonic-clonic and partial seizures usually inhibit sustained high-frequency repetitive firing of action potentials by enhancing Na^+ channel inactivation (Macdonald, 1989; Macdonald and Kelly, 1995). In this study, however, we found that LAG acted by specifically modulating presynaptic N-type Ca^{++} channels; this effect is clearly different from those of other anticonvulsants in terms of mechanism of action. Since LAG has been shown considerable promise in clinical trials (Brodie et al., 1995; Ferrie et al., 1995), the present finding may be of significant help in developing new compounds that have more selective actions.

In summary, we found that LAG inhibits evoked synaptic transmission, most likely by reducing presynaptic Ca^{++} influx in the amygdala. The reduced Ca^{++} influx was largely due to inhibition of presynaptic ω-CgTX-GVIA-sensitive Ca^{++} channels.

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