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Aniracetam reduces glutamate receptor desensitization and slows the decay of fast excitatory synaptic currents in the hippocampus

(non-*N*-methyl-D-aspartate receptor/synapse)

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ABSTRACT **Aniracetam is a nootropic drug that has been shown to selectively enhance quisqualate receptor-mediated responses in *Xenopus* oocytes injected with brain mRNA and in hippocampal pyramidal cells [Ito, I., Tanabe, S., Kohda, A. & Sugiyama, H. (1990) *J. Physiol. (London)* 424, 533–544]. We have used patch clamp recording techniques in hippocampal slices to elucidate the mechanism for this selective action. We find that aniracetam enhances glutamate-evoked currents in whole-cell recordings and, in outside-out patches, strongly reduces glutamate receptor desensitization. In addition, aniracetam selectively prolongs the time course and increases the peak amplitude of fast synaptic currents. These findings indicate that aniracetam slows the kinetics of fast synaptic transmission and are consistent with the proposal [Trussell, L. O. & Fischbach, G. D. (1989) *Neuron* 3, 209–218; Tang, C.-M., Dichter, M. & Morad, M. (1989) *Science* 243, 1474–1477] that receptor desensitization governs the strength of fast excitatory synaptic transmission in the brain.**

A general property of neurotransmitter receptor channels is that in the continuous presence of agonist the response rapidly diminishes. Although this desensitization, which results from a conformational change in the receptors, is readily demonstrated for a number of neurotransmitters, including acetylcholine (1, 2), γ-aminobutyric acid (3), glycine (4), serotonin (5), and glutamate (6–12), a physiological role for desensitization in synaptic transmission has not been established. Rapid perfusion experiments with the non-*N*-methyl-D-aspartate (NMDA) type of glutamate receptor indicate that this receptor desensitizes extremely quickly in the presence of glutamate (6, 7, 10–12) with a time course similar to that of glutamate-mediated synaptic responses. We have investigated whether desensitization could contribute to the decay of excitatory postsynaptic currents (EPSCs) (6, 7). We find that aniracetam, a drug reported to enhance glutamate responses (13), strongly reduces glutamate receptor desensitization. In addition, this drug prolongs the time course and increases the peak amplitude of synaptic currents. These findings suggest that receptor desensitization governs the strength of excitatory synaptic transmission in the brain.

MATERIALS AND METHODS

Experiments were performed on guinea pig hippocampal slices (500 μm) prepared by standard methods (14). After a 1-hr recovery period, slices were placed in a laminar flow recording chamber and superfused with a medium containing (in mM) 125 NaCl, 5 KCl, 4 $MgCl_2$, 4 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, and 10 glucose and equilibrated with 95% O_2/5% CO_2. For all experiments examining synaptic currents or iontophoretic responses in slices, picrotoxin (50–100 μM) and DL-2-amino-5-phosphonovaleric acid (50 μM) were added to the medium to block γ-aminobutyric acid type A ($GABA_A$) receptors and NMDA receptors, respectively. In the majority of experiments examining iontophoretic responses, tetrodotoxin (0.5–1 μM) was included to block sodium-dependent action potentials. Currents were recorded with an Axopatch 1B amplifier from neurons in the CA1 and CA3 pyramidal cell layers and granule cell layer of the dentate gyrus using the "blind" whole-cell recording technique (15, 16). Patch electrodes (tip diameter = 2 μm) contained (in mM) either a CsF (110 CsF, 10 CsCl, 10 Hepes, and 10 EGTA, pH 7.3) or cesium gluconate (117.5 cesium gluconate, 17.5 CsCl, 8 NaCl, 10 Hepes, 2 MgATP, 0.2 GTP, and 0.2–1 EGTA, pH 7.3) internal solution. Iontophoretic electrodes were filled with either glutamate (250 mM, pH 8), quisqualate (10 mM in 150 mM NaCl, pH 8), kainate (5 mM in 150 mM NaCl, pH 8), or domoate, a potent kainate agonist (5 mM in 150 mM NaCl, pH 8). All experiments were performed at room temperature at a holding potential of −80 mV, unless otherwise stated. Room temperature was used to slow synaptic responses, which improved the ability to record these rapid events accurately. Stock solutions of 0.5 M aniracetam or piracetam were dissolved in dimethyl sulfoxide (DMSO) and added directly to the superfusing medium. DMSO alone, diluted to 0.6% in the medium, had no effects. Illustrated traces are averages of three to five responses, except where indicated. Results are presented as mean ± SEM and significance was assessed using Student's *t* test.

To measure glutamate desensitization, currents were recorded from outside-out patches pulled from the somata of pyramidal and granule neurons. Patches were maneuvered within the slice chamber to an apparatus constructed for the rapid application of agonists (6, 8, 17). Briefly, this device consisted of a piezoelectric bimorph element (Vernitron) attached to a segment of theta tubing with a tip diameter of ≈250 μm. Perfusing solutions were gravity-fed to each compartment of the theta tubing. The tip of the patch electrode was positioned ≈100 μm from the tip of the theta tubing, close to the sharp boundary that formed between the two flowing solutions. Solution exchange was achieved by rapidly driving the boundary across the electrode tip with a voltage pulse to the piezoelectric element, remotely triggered at rates of 0.1–0.3 Hz. In general, concentrations of 2 mM agonist were used to generate responses that closely mimicked the rising phase of EPSCs (≈1 ms). The speed of solution exchange was assessed using two methods. The first method involved measuring the change in current generated by the shift in liquid junction potential at the tip of an open patch electrode in response to the rapid exchange of the normal perfusing solution to a solution diluted 10-fold with distilled water. The second method involved studying changes from a

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Abbreviations: NMDA, *N*-methyl-D-aspartate; EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; LTP, long-term potentiation.

solution containing kainate or glutamate to one containing the agonist but with a 10-fold lower concentration of NaCl (18). The shift in driving force for Na^+ and the resulting change in current provide an accurate measure of solution exchange at the membrane surface. Measurements of currents across intact patches or open electrodes showed that the fastest solution exchanges were complete within 2 ms.

RESULTS

Bath application of aniracetam caused a dramatic enhancement in the size of glutamate-evoked responses measured with whole-cell recording techniques from neurons in the hippocampal slice preparation ($n = 10$) (Fig. 1*A*). The effect, which was observed in CA1 and CA3 pyramidal cells as well as dentate granule cells, developed as rapidly as the solution exchange in the recording chamber (1 min) and the response recovered quickly following washout of the drug. On average, the glutamate response was enhanced 209% ± 35% ($P < 0.01$). The ability of aniracetam to enhance these responses in cells dialyzed with a fluoride-containing internal solution, which would be expected to disrupt many metabolic processes (see ref. 14), as well as its rapid action in cell-free patches (see below) favor an allosteric mechanism. Piracetam, a structurally related member of the nootropic class of drugs (19), had no effect on glutamate responses, although these responses were potentiated by the subsequent administration of aniracetam ($n = 3$). Therefore, the enhancing effect is not a common property of all nootropic drugs.

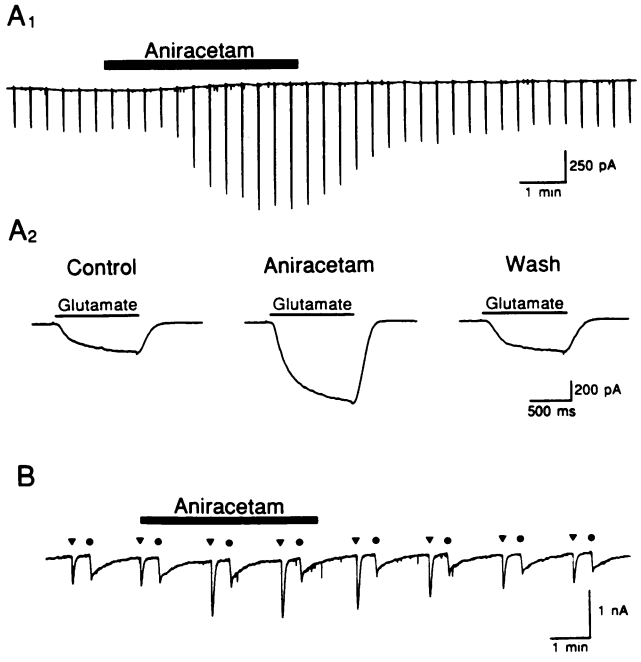

FIG. 1. Aniracetam selectively enhances the responses to glutamate and quisqualate but not kainate in patch-clamped hippocampal neurons. (A_1) Chart record of membrane current from a voltage-clamped CA1 pyramidal cell. Brief iontophoretic pulses of glutamate (70 nA) were applied from an electrode positioned in the stratum radiatum. Aniracetam (2 mM), added to the bathing medium during the time indicated by the bar, increased the size of the glutamate-induced inward current. (A_2) Glutamate responses from the same cell before, during, and after drug application are displayed on a faster time scale. The bar marks the duration of glutamate iontophoresis. (*B*) Record of membrane current from a CA3 neuron. Quisqualate (▼, 50 nA/2-s pulse) and kainate (●, 200 nA/4-s pulse) were applied from a double-barreled iontophoretic electrode placed in the stratum lucidum. Aniracetam (1 mM) caused a marked increase in the response evoked by quisqualate but had no effect on the kainate response.

We have confirmed in the hippocampal slice the finding of Ito *et al.* (13) in *Xenopus* oocytes injected with rat brain mRNA that aniracetam enhances the action of quisqualate ($n = 12$) but not that of kainate ($n = 6$) (Fig. 1*B*) or domoate ($n = 9$). The finding by others that quisqualate can desensitize kainate responses (10, 11) and that all glutamate receptors thus far expressed from cDNA clones respond to quisqualate and kainate (20, 21) suggests that a single receptor responds to both of these agonists. Since the responses to quisqualate and glutamate desensitize whereas those to kainate and domoate do not, we considered the possibility that the selective action of aniracetam might be due to a reduction of desensitization.

To examine this possibility, outside-out patches were made from the somata of pyramidal and granule cells in the hippocampal slice. Drugs were rapidly delivered to the patch via an apparatus that allowed the switching of perfusing solutions within 2 ms. The peak amplitudes of responses to glutamate ranged from 25 to 200 pA. These sizable macroscopic currents suggested a high density of somatic glutamate receptors and precluded the analysis of single-channel properties. The glutamate and quisqualate responses desensitized with time constants as short as 4.5 ms and with an average of 16.8 ± 8 ($n = 9$), values that are limited by the speed of the solution change. In the presence of aniracetam the rate of decay of these responses was markedly slowed (215% ± 43%, $n = 9$) ($P < 0.01$) and the peak amplitude of the responses increased (51% ± 11%, $n = 10$) ($P < 0.01$). Kainate responses were unaffected (Fig. 2*B*). Although these results do not exclude a direct effect of aniracetam on channel open time, its effect on the rapid inactivation of the response in the continued presence of glutamate indicates that the major action of this drug is to reduce receptor desensitization.

Given the dramatic effects of aniracetam on responses to glutamate we next examined its effects on synaptic currents at Schaffer collateral-commissural synapses in CA1 ($n = 5$), mossy fiber synapses in CA3 ($n = 3$), and perforant path synapses in the dentate gyrus ($n = 5$). Aniracetam had two very consistent effects at these synapses. It prolonged the rate of decay and increased the peak amplitude of the EPSC (Fig. 3*A*). The average increase in the decay time constant was 90% ± 9% ($n = 10$) ($P < 0.01$), whereas the peak current increased 116% ± 14% ($n = 10$) ($P < 0.01$). There was no obvious difference in the effect of aniracetam on the different types of synapses. The two effects of aniracetam had similar dependencies on concentration and similar time courses of action, suggesting that they may be due to a common mechanism of action. These effects on synaptic currents

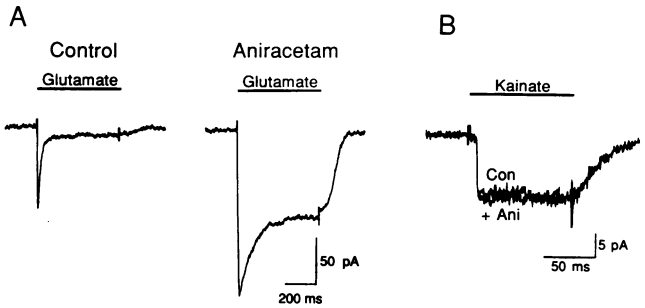

FIG. 2. Aniracetam reduces glutamate receptor desensitization in outside-out patches. (*A*) Responses to a 500-ms glutamate application in a CA1 outside-out patch. Macroscopic currents were recorded in the absence and presence of aniracetam (2 mM). Glutamate (2 mM) was applied for the time marked by the bar. Aniracetam caused a 4-fold reduction in the rate of desensitization and increased the steady-state current level to a much greater extent than the peak response. (*B*) Aniracetam (2 mM) had no effect on the response to the rapid perfusion of kainate (2 mM, $n = 2$). The response in aniracetam (+ Ani) is superimposed over the control (Con) response.

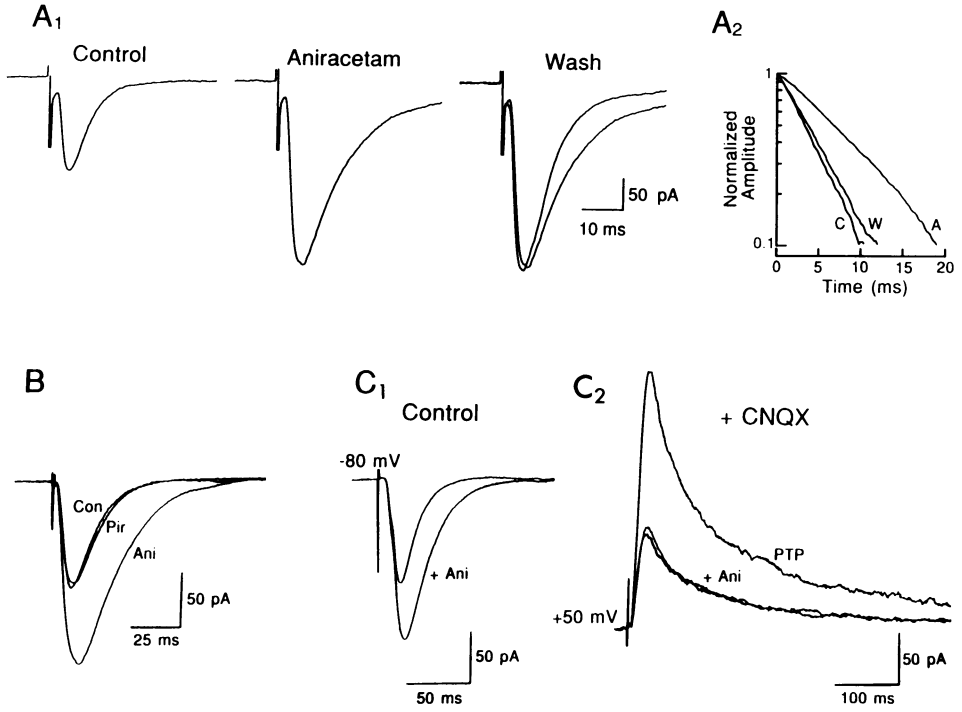

FIG. 3. Aniracetam increases the amplitude and prolongs the time course of non-NMDA receptor-mediated EPSCs. (A_1) Voltage clamp recordings of EPSCs in a granule cell before (Control) and during (Aniracetam) the addition of aniracetam (2 mM) to the superfusing medium. After washout of the drug, the stimulus strength was increased to match the peak amplitude of the EPSC in the presence of aniracetam. These responses are shown superimposed (Wash) and emphasize that aniracetam slows the time course of the EPSC. (A_2) Semilogarithmic plot of the decay time courses of EPSCs recorded in this cell before (C), during (A), and after (W) treatment with the drug. The synaptic currents were well-fitted by single exponential functions with time constants of 4.5 (C), 10.3 (A), and 5.3 (W) ms. (B) Synaptic currents in a CA1 cell (Con) were unaffected by the addition of piracetam (3 mM) to the bath (Pir), whereas a subsequent application of aniracetam (Ani, 2 mM) caused a large increase in the size of the EPSC. (C) Aniracetam has no effect on the NMDA receptor-mediated EPSC. (C_1) EPSCs from a CA1 cell were first recorded at −80 mV and were increased in size when the superfusing solution was changed to one containing aniracetam (2 mM) (+ Ani). After washout of the drug, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 15 μM) was added to the bath and completely blocked the EPSC at this potential (not shown). (C_2) The cell was then depolarized to a holding potential of +50 mV and the stimulus strength was increased to generate NMDA receptor-mediated EPSCs. Aniracetam had no effect on these synaptic currents and the responses before and after (+ Ani) treatment are superimposed. However, the response did increase during the expression of post-tetanic potentiation induced by a 100 Hz/1-s tetanus, as shown by the average of the first two EPSCs immediately following the tetanus (PTP). Synaptic currents were evoked at 0.1 Hz from bipolar stainless steel electrodes placed in the stratum radiatum for CA1 recordings and the perforant path for recordings from dentate granule cells.

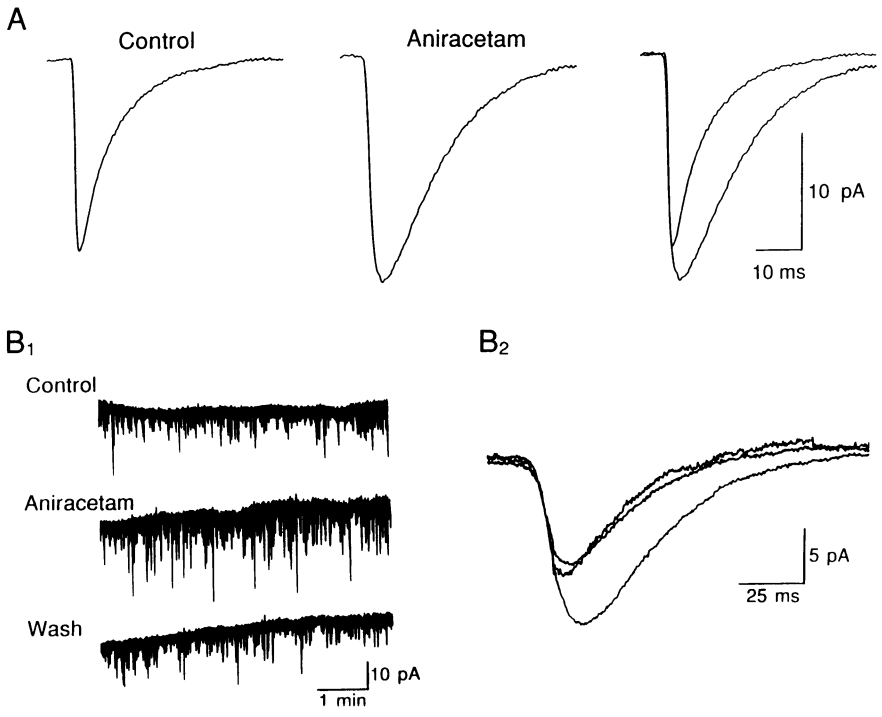

FIG. 4. Aniracetam prolongs the time course and increases the amplitude of mEPSCs. (A) Averages of 50 mEPSCs from a granule cell before (Control) and during the superfusion of 3 mM aniracetam (Aniracetam) and the superimposition of these records. (B_1) Chart records of mEPSCs in a CA1 neuron held at −80 mV. Aniracetam (2 mM) caused an increase in the amplitude of mEPSCs in this cell, which reversed upon washout of the drug. (B_2) Twenty mEPSCs recorded from this cell before, during, and after treatment with aniracetam were aligned and averaged.

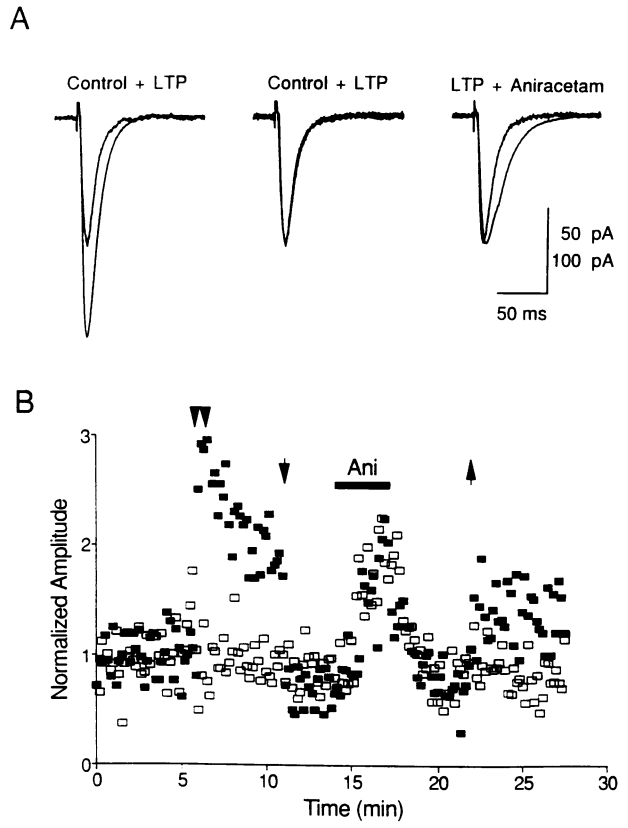

FIG. 5. Comparison of the effect of LTP and aniracetam on the EPSC. (*A*) Comparison of the effect of LTP and aniracetam on the decay of the EPSC. (*Left*) Superimposition of the control response onto the response elicited 10 min after pairing 20 EPSCs at a holding potential of −10 mV. The EPSC showed an approximate 3-fold potentiation (the lower gain refers to the LTP trace). (*Center*) Superimposition of the control response with the response obtained after LTP and after the stimulus had been turned down (calibration = 50 pA). (*Right*) Superimposition of the control response with the response elicited in the presence of aniracetam (2 mM). The response in the presence of aniracetam is scaled down to the amplitude of the control response. (*B*) Graph of an experiment comparing the effects of LTP and aniracetam on the amplitude of EPSCs. At the time of the double arrowhead a tetanus (100 Hz, 1 s repeated twice) was delivered to one of two independent pathways while the cell was held at −10 mV. The stimulus strength of the potentiated pathway (filled squares) was then reduced to match the amplitude of the independent control pathway (downward arrow). After the response to aniracetam (2 mM) had recovered, the stimulus to the tetanized pathway was returned to its original level (upward arrow).

would be expected to greatly augment synaptic potentials recorded in current clamp, as reported previously (13). Piracetam, which had no effect on glutamate responses (see above), also had no effect on EPSCs (n = 4) (Fig. 3*B*).

Synaptically released glutamate acts on NMDA and non-NMDA receptors, which are colocalized at single excitatory synapses (22, 23). In contrast to the enhancing action of aniracetam on non-NMDA receptor-mediated EPSCs, no effect was detected on the NMDA component of the EPSC. This is shown in Fig. 3*C*, in which aniracetam was first shown to exert its normal effect at a holding potential of −80 mV. 6-Cyano-7-nitroquinoxaline-2,3-dione was then added to the superfusion medium to block the non-NMDA component of the EPSC and the holding potential was shifted to +50 mV to alleviate the Mg^{2+} block of the NMDA synaptic current. Aniracetam had no effect on the NMDA receptor-mediated EPSC, although posttetanic potentiation, which increases transmitter release, clearly enhanced this current (n = 4). The lack of effect of aniracetam on the NMDA component of the EPSC favors a postsynaptic mechanism for its action on the non-NMDA component.

To further characterize the site at which aniracetam alters the EPSC, we examined its action on miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin to block action potential-dependent synaptic events. Aniracetam prolonged the time course of mEPSCs in all cells examined, and in four of the six cells it caused an increase in their peak amplitude (Fig. 4).

We have also examined whether long-term potentiation (LTP), a process in which brief repetitive synaptic stimulation paired with postsynaptic depolarization results in a long-lasting enhancement of synaptic transmission, and aniracetam might share a common mechanism. In contrast to the effect of aniracetam on the time course of the EPSC, LTP had no obvious effect on the decay of the EPSC (n = 6) (Fig. 5*A*). It has recently been reported that the action of aniracetam on the amplitude of field excitatory postsynaptic potentials is less in pathways expressing LTP (24). Fig. 5*B* shows an example of an experiment with whole-cell recording in which a comparison between LTP and the action of aniracetam on the amplitude of the EPSC has been made in the same cell. In this experiment the responses to two independent pathways converging onto the same cell were recorded. Tetanic stimulation to one pathway, paired with postsynaptic depolarization, caused a large increase in the size of the EPSC. However, when the stimulus strength of the pathway expressing LTP was reduced so that the peak amplitude of the synaptic response was the same as that in the independent control pathway, aniracetam had similar effects on both pathways. In six cells aniracetam increased the size of the EPSC in the control pathway 108% ± 19%, and in the pathway expressing LTP aniracetam increased the EPSC 126% ± 19%. The difference was not significant.

DISCUSSION

We have used rapid perfusion techniques and outside-out membrane patches from hippocampal slices to elucidate the mechanism for the enhancing action of aniracetam on glutamate responses, which we and others have found to be rapidly desensitizing (6–12, 25). Aniracetam markedly slowed the kinetics of the glutamate response by reducing receptor desensitization. We therefore used this drug to test the proposal (6) that glutamate receptor desensitization might limit excitatory synaptic transmission. Indeed, aniracetam produced nearly a doubling in the decay time constant and amplitude of EPSCs. However, concanavalin A, a lectin that reduces glutamate receptor desensitization, does not prolong synaptic responses in cultured neurons (12). This lack of effect might be due to the poor access of concanavalin A to the synaptic region. Indeed, the lectins concanavalin A and wheat germ agglutinin have no effect on glutamate or synaptic responses recorded from pyramidal cells in the slice. Alternatively, concanavalin A might affect a different component of glutamate receptor desensitization that is not involved in the decay of the EPSC. Although at the macroscopic level aniracetam appeared to act primarily by blocking desensitization, it cannot be ruled out that at the single-channel level aniracetam might alter channel kinetics by an action independent of desensitization. Single-channel studies will be required to address this issue.

The ability of aniracetam to increase the amplitude of the responses to the rapid application of glutamate and EPSCs might arise from a number of mechanisms. One possibility is that glutamate receptors might enter a desensitized state during the onset of the glutamate response and the rising phase of the EPSC. Therefore, the peak of these macroscopic currents would be limited by desensitization. Alternatively, it has been shown that glutamate concentrations in the same range as that detected in the extracellular fluid (2–4 μM) (26,

27) can desensitize glutamate responses and mEPSCs (6). Thus, the increase in amplitude of the EPSC might reflect the removal of background desensitization, thereby increasing the number of functional receptors.

Since aniracetam causes a large enhancement of EPSCs we examined the possibility that it might share the same mechanism as LTP. However, in contrast to the action of aniracetam, no obvious change in the decay time constant of the EPSC was seen with LTP. In addition, though we cannot rule out subtle interactions between the action of aniracetam and LTP (see ref. 24), it is clear from our results with whole-cell recording that the major action of aniracetam on synaptic strength is distinct from LTP.

In this study, we have described the modulation of glutamate receptor desensitization by an allosteric mechanism. However, the desensitization of a variety of other neurotransmitter receptors has been shown to be regulated by protein phosphorylation (28). An intriguing possibility is that protein phosphorylation, induced by other neurotransmitter receptors, may also modulate glutamate receptor desensitization and thereby control the strength of excitatory synaptic transmission.

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