Journal of Physiology (1990), **424**, *pp.* 533–543
With 5 figures
Printed in Great Britain

ALLOSTERIC POTENTIATION OF QUISQUALATE RECEPTORS BY A NOOTROPIC DRUG ANIRACETAM

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(*Received 2 October 1989*)

SUMMARY

1. Allosteric potentiation of the ionotropic quisqualate (iQA) receptor by a nootropic drug aniracetam (1-*p*-anisoyl-2-pyrrolidinone) was investigated using *Xenopus* oocytes injected with rat brain mRNA and rat hippocampal slices.

2. Aniracetam potentiates the iQA responses induced in *Xenopus* oocytes by rat brain mRNA in a reversible manner. This effect was observed above the concentrations of 0·1 mM. Kainate, *N*-methyl-D-aspartate and γ-aminobutyric acid responses induced in the same oocytes were not affected.

3. The specific potentiation of iQA responses was accompanied by an increase in the conductance change of iQA and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) responses, but the affinity of receptors for agonist and the ion-selectivity of the channels (reversal potentials) were not changed.

4. Aniracetam reversibly potentiated the iQA responses recorded intracellularly from the pyramidal cells in the CA1 region of rat hippocampal slices. The excitatory postsynaptic potentials (EPSPs) in Schaffer collateral–commissural-CA1 synapses were also potentiated by aniracetam.

5. Population EPSPs recorded in the mossy fibre-CA3 synapses as well as Schaffer–commissural synapses were also potentiated by aniracetam. The amplitudes of the potentiation were not changed by the formation of long-term potentiation.

INTRODUCTION

Glutamate (Glu) is believed to be a major excitatory neurotransmitter in the mammalian central nervous system, and the properties of its receptors may provide critical keys for the understanding of the molecular mechanisms of the brain functions such as memory and learning. For instance, the functional properties of Glu receptors of *N*-methyl-D-aspartate (NMDA) type, for example, high permeability to Ca^{2+} ions (MacDermott, Mayer, Westbrook, Smith & Barker, 1986; Ascher & Nowak, 1988), voltage-dependent blockade by Mg^{2+} ions (Mayer, Westbrook &

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Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984) or allosteric regulation by glycine (Johnson & Ascher, 1987), provide a fundamental basis for molecular mechanisms of long-term potentiation (LTP) (Collingridge & Bliss, 1987; Kauer, Malenka & Nicholl, 1988; Muller, Joly & Lynch, 1988). In contrast to this, however, the functional properties of non-NMDA-type Glu receptor channels are poorly understood, although the regulatory mechanisms of their functions must be critically important, because, for instance, it has recently been shown that it is the non-NMDA-type Glu receptor channels that are potentiated upon the formation of LTP (Kauer *et al.* 1988; Muller *et al.* 1988; Davies, Lester, Reymann & Collingridge, 1989).

In the present study, we examined the effects of a nootropic drug aniracetam, which is reported to affect learning and memory processes (cognitive activator) (Cumin, Bandle, Gamzu & Haefely, 1982; Vincent, Verderese & Gamzu, 1985), on the function of different glutamate receptor channels, and have found that the drug specifically potentiated the responses mediated by the quisqualate (QA) receptor channel to more that three times control, without affecting the responses of the NMDA or kainate receptor channels. The results provide the first clear evidence for the readily reversible allosteric potentiation of non-NMDA receptor channels.

METHODS

Experimental procedures

All experiments using *Xenopus* oocytes were performed as described before (Sugiyama, Ito & Hirono, 1987; Sugiyama, Ito & Watanabe, 1989).

Hippocampal slices were prepared from male Wistar rats (270–320 g) as described previously (Ito, Okada & Sugiyama, 1988; Okada, Yamagishi & Sugiyama, 1989). Intracellular electrodes were filled with 3 M-KCl (about 30 MΩ), and only cells having resting membrane potentials of < -60 mV, a spike overshoot of > 20 mV, and membrane impedance of > 20 MΩ were examined. Extracellular electrodes were filled with 0·9% NaCl, and were placed either in the stratum radiatum of CA1 regions or the stratum lucidum of CA3 regions. To evoke synaptic transmission, stimuli were given by a bipolar stainless-steel electrode placed either in the stratum radiatum in the CA1 regions (to stimulate Schaffer collateral–commissural pathways) or in the dentate granular cell layer (to stimulate mossy fibres). Data were collected using a microcomputer (NEC PC-9801 VX2).

Solutions

For *Xenopus* oocytes experiments the compositions of solutions (in mM) were as follows. Incubation medium (MBS): NaCl, 88; KCl, 1·0; $CaCl_2$, 0·41; $Ca(NO_3)_2$, 0·33; $MgSO_4$, 0·82; $NaHCO_3$, 2·4; Tris-HCl, 7·5 (pH 7·6). Ca^{2+}-free MBS: MBS devoid of $CaCl_2$ and $Ca(NO_3)_2$. Normal Ringer solution: NaCl, 90; KCl, 2; $CaCl_2$, 2; Tris-HCl, 10 (pH 7·6). For hippocampal slices experiments solutions (in mM) were as follows. Krebs Ringer solution: NaCl, 117; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 25; glucose, 11·5. Low-Ca^{2+} Ringer solution: the same as Krebs Ringer solution except for $CaCl_2$, 0·2; $MgCl_2$, 6·0. Ca^{2+}-free Ringer solution: Krebs Ringer solution devoid of $CaCl_2$, $NaHCO_3$ and glucose.

Materials

Female frogs (*Xenopus laevis*) were obtained from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan). Male Wistar rats were from Charles River Japan Inc. (Atsugi, Japan).

Chemicals were obtained from the following sources. Quisqualate, kainate: Sigma; γ-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA): Research Biochemicals; NMDA: Cambridge Research Biochemicals; γ-aminobutyric acid (GABA), ethyleneglycol-bis-(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), dimethylsulphoxide (DMSO): Wako, Japan.

Aniracetam was synthesized in our laboratory using the methods of European patent 5, 143. The melting point of the preparation was 122 ± 0·5 °C. Aniracetam was dissolved in DMSO and added to the Ringer solutions. The final concentration of the DMSO was 0·15%.

RESULTS

Studies on Xenopus oocytes

Potentiation of iQA responses

A few days after the injection of poly(A)$^+$-RNA isolated from rat brains, oocytes acquired responsiveness to several excitatory and inhibitory amino acids (Gundersen,

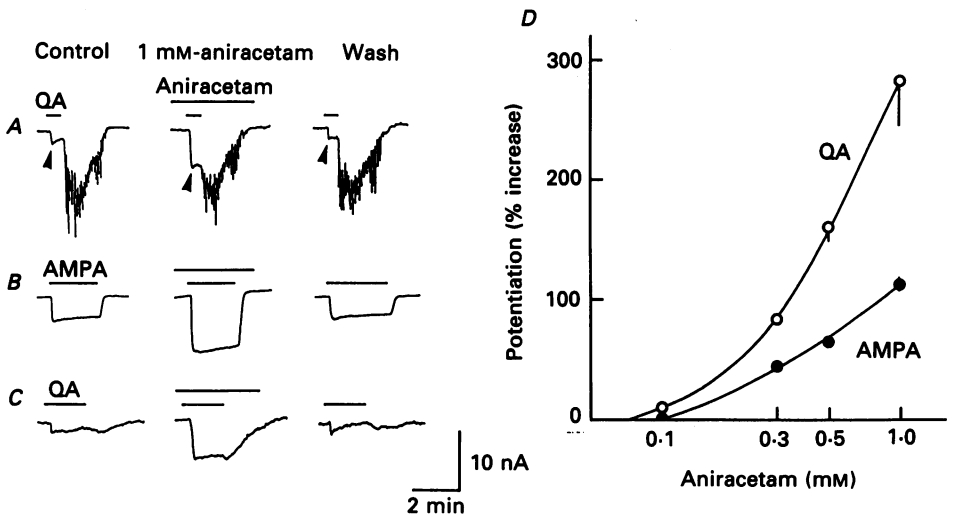

Fig. 1. Potentiating effects of aniracetam on current responses mediated by QA receptors in *Xenopus* oocytes injected with rat brain mRNA. *A*, QA evoked ionotropic responses (arrow-heads), which were followed by the oscillatory responses mediated by the metabotropic Glu receptors. The former was selectively potentiated by aniracetam. *B*, AMPA evoked pure ionotropic responses which were potentiated by aniracetam. In *C*, the oocyte was injected with EGTA (0·2 nmol) a few minutes before the measurement. *D*, dose dependence of the potentiating effect of aniracetam on iQA or AMPA responses. Concentrations of QA and AMPA were 20 μM. Each point represents a mean ± S.E.M. for $n = 3$–5.

Miledi & Parker, 1984; Sugiyama *et al.* 1987; Verdoorn, Kleckner & Dingledine, 1987; Snutch, 1988). Under the voltage clamp condition (−60 mV), bath application of QA (20 μM) elicited characteristic current responses as shown in Fig. 1*A*. The QA responses induced in *Xenopus* oocytes are composed of two different components (Gunderson *et al.* 1984; Sugiyama *et al.* 1987; Hirono, Ito, Yamagishi & Sugiyama, 1988). The smooth inward currents with little decay are mediated by the ionotropic type of QA receptors (iQA receptors) (Hirono *et al.* 1988). The oscillatory responses with long latency are mediated by the metabotropic type of Glu receptor which functions through the activation of a G-protein (Sugiyama *et al.* 1987). Aniracetam (1 mM) reversibly potentiated the smooth component of the QA responses (Fig. 1*A*). For the quantitative analysis of this potentiation, it is desirable to eliminate the

oscillatory component of QA responses. This was accomplished by the use of AMPA, which is known to be a specific agonist for the iQA receptors but is not effective for metabotropic Glu receptors (Sugiyama *et al.* 1989), or by the intracellular injection of EGTA (Sugiyama *et al.* 1987). As shown in Fig. 1*B* and *C*, aniracetam reversibly

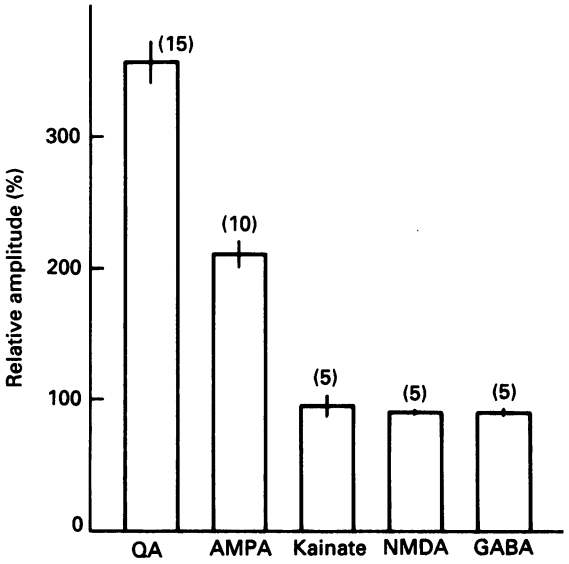

Fig. 2. Specificity of the aniracetam effect on amino acid responses in mRNA-injected oocytes. The amplitudes of current responses to various amino acids in the presence of 1 mM-aniracetam are shown relative to its absence as mean ± S.E.M. (*n*). Concentrations are: QA and AMPA, 20 μM; kainate and GABA, 100 μM; NMDA, 100 μM together with 10 μM-glycine.

potentiated the AMPA responses and the iQA responses in the EGTA-injected oocytes. In subsequent studies we thus used EGTA-injected oocytes when QA responses were analysed. Potentiation of the iQA response by aniracetam was observed at the concentration of 0·1 mM and increased with its dose (Fig. 1*D*). The effect reached its plateau level instantaneously, because the same extent of potentiation was observed whether aniracetam was applied simultaneously with or a few minutes prior to QA.

Specificity

To test the specificity of the aniracetam potentiation, we examined the effect of aniracetam on kainate, NMDA and GABA responses induced in *Xenopus* oocytes. As shown in Fig. 2, aniracetam (1 mM) potentiated the responses to QA and AMPA without significant effects on kainate, NMDA and GABA responses.

Mode of action of the potentiation

Figure 3*A* shows the *I–V* relations of iQA and AMPA responses in the absence or presence of 1 mM-aniracetam. Aniracetam caused increases in slope conductance of iQA and AMPA responses without affecting their reversal potentials. As shown in Fig. 3*B*, potentiation of AMPA responses by aniracetam was accompanied by increases in the maximum amplitude of the responses, but the affinity of receptors

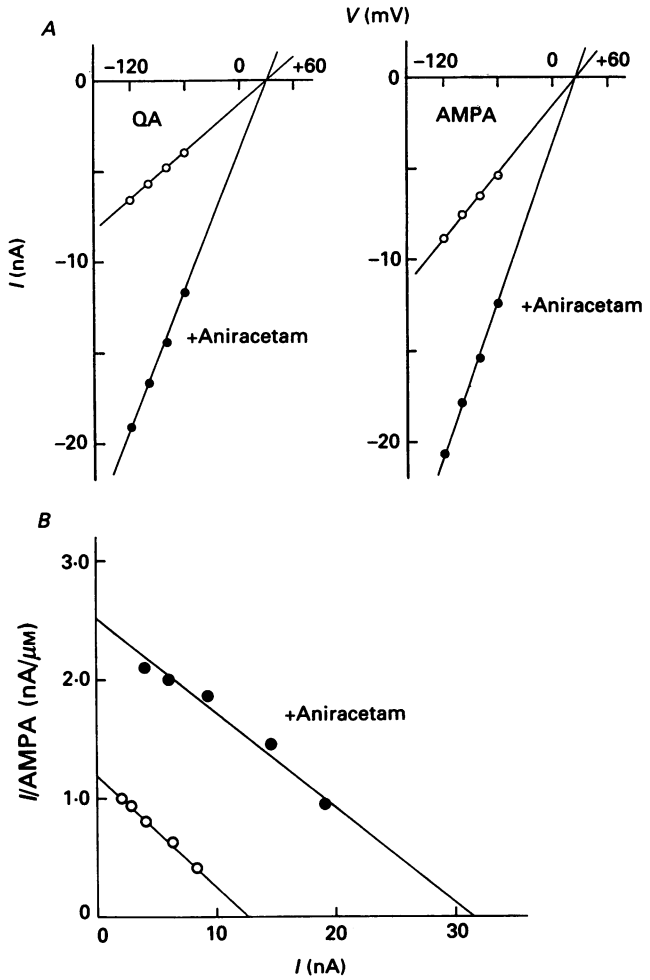

Fig. 3. *I–V* relationship (*A*) and Scatchard analysis (*B*) of the aniracetam effect on the QA and AMPA responses in mRNA-injected oocytes. Aniracetam (1 mM) potentiated the QA- or AMPA-activated conductance without affecting the reversal potentials (*A*) or the agonist affinity (*B*). In *A*, the reversal potentials were estimated by extrapolating the data obtained in a hyperpolarized potential range, and may be somewhat more positive than the 'real' ones because of the weak curvature of the *I–V* curves observed in more depolarized ranges (Hirono *et al.* 1988). In *B*, the apparent dissociation constants were 10·4 and 12·4 μM in the absence and presence of aniracetam, respectively, while the maximum currents were 12·5 and 31·5 nA in the absence and presence of aniracetam, respectively. Concentrations of QA and AMPA were 20 μM.

for AMPA was not changed. Thus, in *Xenopus* oocytes injected with rat brain mRNA, aniracetam enhances the conductance increase of iQA receptor channels in an allosteric manner, without affecting the agonist affinity or ion selectivity.

Aniracetam analogues

Other structurally related compounds were also examined. 1-*p*-Anisoyl-pyrrolidine (1 mM) was found to potentiate iQA responses of oocytes by 50%, whereas 2-pyrrolidinone or 2-oxo-1-pyrrolidineacetamide (piracetam) was ineffective.

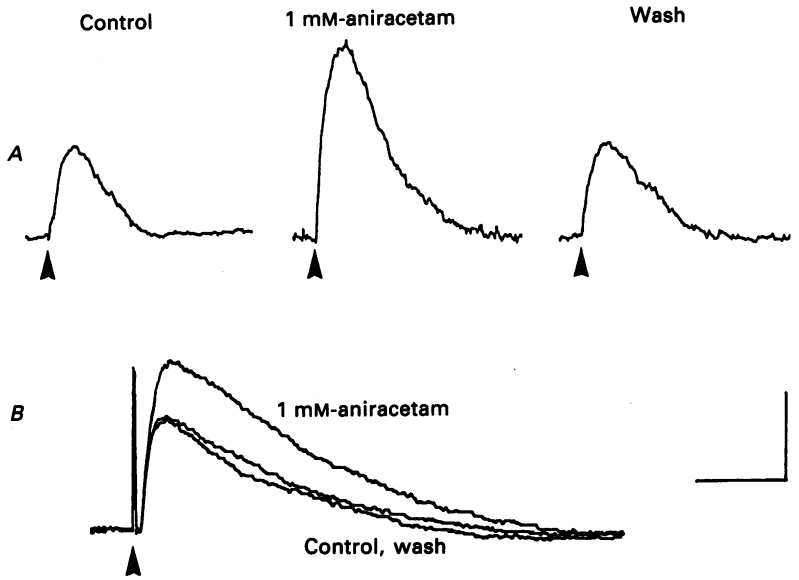

Fig. 4. Enhancing effects of aniracetam (1 mM) on QA responses (*A*) and EPSPs (*B*) measured in CA1 pyramidal neurones of rat hippocampal slices. *A*, QA was applied from a micropipette by pressure pulses (100 ms at arrow-heads). Quisqualate (30 μM) was dissolved in Ca^{2+}-free Ringer solution. *B*, EPSPs evoked by stimulations given to Schaffer collateral–commissural fibres (arrow-head). Each trace is an average of five consecutive trials. Calibrations: 8 mV and 6 s (*A*), and 10 mV and 10 ms (*B*).

TABLE 1. Potentiating effects of aniracetam before and after the formation of long-term potentiation (LTP) in hippocampal slices

	Aniracetam potentiation		
Region	Before LTP* (%)	Ratio (after/before)†	LTP‡ (%)
CA1	26±3 (5)	0·75±0·13 (4)§	58±5 (4)
CA3	29±8 (7)	1·01±0·10 (7)	76±10 (7)

Potentiations were estimated from experiments like those shown in Fig. 5 by comparing the amplitudes of field potentials measured in stratum radiatum of CA1 regions or stratum lucidum of CA3 regions. Stimulations were given as described in Methods. * Percentage increases in amplitudes by aniracetam (1 mM) measured before the formation of LTP. Mean±S.E.M. (*n*). † Ratios of percentage increases in amplitudes by aniracetam (1 mM) measured after LTP to those before LTP. Mean±S.E.M. (*n*). ‡ Percentage increases in amplitudes measured 30 min after tetanic stimulation given as described in Fig. 5 legend. Mean±S.E.M. (*n*). §$P > 0·1$ by paired *t* test.

Studies on hippocampal slices

Effects on iQA potentials of pyramidal neurones

The potentiating effects of aniracetam were also observed in rat hippocampal neurones. Intracellular recordings were made from CA1 pyramidal cells. Figure 4*A* shows a typical response of a pyramidal cell to QA (30 μM) applied by a pressure

ejection pulse of 100 ms. To inhibit the synaptic transmission and depolarization-induced transmitter releases, slices were perfused with low-Ca^{2+} Krebs Ringer solution. Bath application of aniracetam reversibly potentiated the QA responses. The average amplitude of the QA responses in aniracetam (1 mM) was 210±20% of the control (mean±S.E.M., $n = 6$).

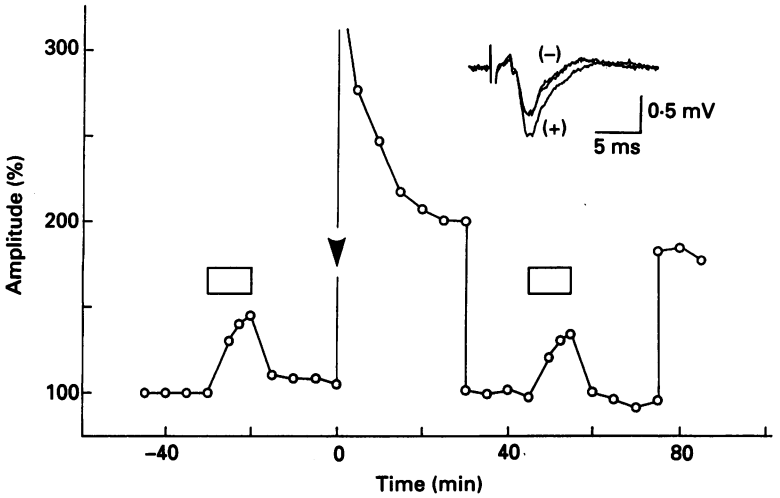

Fig. 5. An example of changes in the amplitudes of field potentials recorded in stratum lucidum of CA3 regions of rat hippocampal slices. The stimulation was given to the granular cell layer. At the arrow-head a tetanic stimulation of 100 Hz for 1 s was given twice (20 s apart), with the same strength as test pulses. The open boxes indicate the periods during which aniracetam (1 mM) was perfused. During the period of $t =$ 30–75 min, the strength of stimulation was reduced so that the amplitude of the field potential was approximately the same as the pre-tetanic original level. Inset: examples of the field potentials. The traces marked (−) were recorded before application of aniracetam and after 30 min wash, and the trace marked (+) was recorded when aniracetam (1 mM) was perfused for 10 min. Each trace is an average of five consecutive trials.

Effects on synaptic potentials of CA1 pyramidal neurones

We then tested the effects of aniracetam on synaptic transmission. A bipolar stimulating electrode was placed in the stratum radiatum of the CA1 area to activate the Schaffer collateral–commissural fibres, and the stimulation current was adjusted to a level at which the EPSP amplitude could be reliably measured but was subthreshold for spike discharge. As shown in Fig. 4*B*, 1 mM of aniracetam reversibly potentiated the amplitude of postsynaptic potentials (EPSPs) recorded intracellularly from CA1 neurones. The average potentiation of EPSPs in aniracetam (1 mM) was 42±5% of the control (mean±S.E.M., $n = 5$). The resting membrane potential and membrane impedance were not affected by the application of aniracetam. The similar potentiating effects of aniracetam were also detected by field potential measurement (Table 1), although the extent of potentiation (26±3%) was slightly smaller than that measured intracellularly (the reason is not known). These results indicate that aniracetam potentiates the iQA receptors existing in the brain and produces remarkable facilitation of the native synaptic transmission.

Effects on synaptic transmission of mossy fibre synapses

It has been reported that the mossy fibre terminal zones in CA3 regions are particularly dense in kainate binding sites compared to AMPA binding sites, whereas AMPA sites are highly concentrated in CA1 regions (Cotman, Monaghan, Ottersen & Storm-Mathisen, 1987). This led to a hypothesis that mossy fibre synapses are mainly mediated by kainate receptors whereas Schaffer–commissural synapses are mediated by iQA receptors. However, the direct functional evidence for this hypothesis is still missing due to the lack of specific antagonists which can distinguish between theses two receptors. Since aniracetam can distinguish, at least in the oocytes, kainate and iQA responses, we tried to apply aniracetam as a pharmacological tool to analyse the properties of mossy fibre–CA3 pyramidal cell synapses. As shown in Table 1, aniracetam (1 mM) reversibly potentiated the mossy fibre synaptic transmission measured by field synaptic potentials. The extent of the potentiation ($29 \pm 8\%$) was very similar to that obtained for Schaffer–commissural synapses in CA1 regions (Table 1). This suggests that the iQA receptors may be involved in the mossy fibre synaptic transmission to a similar extent to that in Schaffer–commissural synapses.

Aniracetam potentiation and long-term potentiation

It has recently been shown that iQA receptor responses were potentiated after the formation of LTP in Schaffer–commissural synapses in hippocampal CA1 regions (Kauer *et al.* 1988; Muller *et al.* 1988; Davies *et al.* 1989). To examine the relationship between the potentiation by aniracetam and that by LTP, we compared the effects of aniracetam before and after the formation of LTP. An example of such experiments is shown in Fig. 5. As shown in Table 1, aniracetam potentiated the synaptic transmission both before and after the formation of LTP to a similar extent, either at Schaffer–commissural synapses or at mossy fibre synapses. These results indicate that the potentiation by aniracetam and LTP may most likely be independent phenomena.

DISCUSSION

Allosteric potentiation of iQA receptors

Although functional modulations of NMDA receptors or $GABA_A$ receptors by different ligands are well known (Johnson & Ascher, 1987; Olsen & Venter, 1987; Bormann, 1988; Stelzer & Wong, 1989), little has been known about the modulation of non-NMDA-type Glu receptors. In this report we provided direct evidence for the allosteric potentiation of iQA receptors. The mode of potentiation is similar to that of NMDA receptors by glycine (Johnson & Ascher, 1987) and GABA receptors by glutamate (Stelzer & Wong, 1989), and may be different from that of GABA receptors by benzodiazepine (Olsen & Venter, 1987; Bormann, 1988), in that the latter was accompanied by a change in the agonist affinity of the receptors. Thus it is most likely that aniracetam modifies the properties of QA-activated cation channels, such as unit conductance and/or gating kinetics. It may also be possible that aniracetam causes transformation of non-functional receptor–channel complexes into functional ones.

It has recently been reported that concanavalin A (ConA) selectively potentiates iQA receptors (Mayer & Vyklicky, 1989). The features of the ConA potentiation are that it develops slowly and irreversibly, and that it may require some intra- and/or intermolecular cross-linkings by the multivalent lectins (Mayer & Vyklicky, 1989). In contrast to this, the effects of aniracetam are instantaneous and readily reversible. Thus the effects of aniracetam, a ligand much smaller than ConA, may most likely be exerted by its direct interaction with the iQA receptor itself. Although the precise mechanism of potentiation has yet to be elucidated, these results suggest that ConA potentiation and aniracetam potentiation may most likely be produced by different mechanisms.

The potentiating effects of aniracetam were specific for iQA-activated channels, and were not exerted on kainate- or NMDA-activated channels, indicating that aniracetam can serve as a useful pharmacological tool to distinguish iQA receptor-mediated responses from other glutamatergic responses. For instance, there has been some controversy over whether iQA and kainate receptors are two distinct receptors or not (Kiskin, Krishtal & Tsyndrenko, 1986; O'Brien & Fischbach, 1986; Verdoorn & Dingledine, 1988; Rassendren, Lory, Pin, Bockaert & Nargeot, 1989), and our observations on aniracetam effects are more readily explained by the two separate receptors hypothesis (Hirono *et al.* 1988).

The concentrations of aniracetam needed for the potentiation of iQA receptors seem to be significantly higher than the effective concentration of aniracetam in the brain which produces cognitive enhancement. Therefore, it seems unlikely that the reported nootropic (cognition-enhancing) effects of aniracetam (Cumin *et al.* 1982; Vincent *et al.* 1985) are due to its allosteric effects described here.

Characterization of the synaptic transmission in mossy fibre-CA3 synapses

It has been hypothesized that mossy fibre synaptic transmission may be largely mediated by kainate receptors whereas Schaffer–commissural synaptic transmission is mediated by iQA receptors, mainly based on the regional distribution of kainate and QA binding sites (Cotman *et al.* 1987). Our results, however, showed that the extent of aniracetam potentiation in mossy fibre synapses is comparable to that in CA1 Schaffer–commissural synapses, and may mean that the pharmacological properties of the two types of synapses could be very similar to each other. This is not readily consistent with the hypothesis mentioned above, provided that the specificity of potentiating effects of aniracetam is the same in hippocampal neurones as that observed in mRNA-injected oocytes. More extensive studies on the potentiating effect of aniracetam on iQA receptors are required on the one hand, but re-examination of the significance and identity of the binding sites, on the other hand, may also be necessary to settle the issue.

We thank Mr Kazumi Morikawa for his technical assistance.

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