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Inhibition of [^{3}H]γ-aminobutyric acid release by kainate receptor activation in rat hippocampal synaptosomes

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Abstract

We studied the modulation of γ-aminobutyric acid (GABA) release by activation of kainate receptor in rat whole hippocampal synaptosomes. Kainate (10–300 μM) inhibited [^{3}H]GABA release in a concentration-dependent manner with an EC_{50} of 25 μM. This effect of kainate (30 μM) was prevented by the ionotropic non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and by the selective kainate receptor antagonist 5-nitro-6,7,8,9-tetrahydrobenzo(*g*)indole-2,3-dione-3-oxime (NS-102, 10 μM), but not by the selective non-competitive AMPA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466, 100 μM). Other kainate receptor agonists, such as domoic acid (0.3–10 μM) and (2*S*,4*R*)-4-methylglutamic acid (MGA, 0.3–3 μM), also inhibited [^{3}H]GABA release in a concentration-dependent manner with EC_{50} values of 4.0 μM and 0.90 μM, respectively, whereas α-amino-3-hydroxy-5-methyl-4-oxazolepropionate (AMPA, 10–100 μM) was devoid of effect. These inhibitory effects of both domoic acid (3 μM) and MGA (1 μM) were antagonized by CNQX (10 μM). These results indicate that GABA release can be modulated directly by presynaptic high-affinity kainate heteroreceptors.

Keywords: Kainate; Hippocampus; GABA (γ-aminobutyric acid) release; Domoic acid; Glutamate; Synaptosome

1. Introduction

The hippocampus is a central nervous system area where kainate receptors have an important role in both physiological and pathological situations. Thus, kainate administration promotes epileptic seizures and hippocampal pyramidal cell loss in rats (Gaiarsa et al., 1994; Coyle, 1983), and the selective kainate receptor agonist, domoic acid (e.g., Egebjerg et al., 1991), can induce severe neurological dysfunction in humans (Teitelbaum et al., 1990). Kainate and domoic acid may produce their effects either by activation of postsynaptic ionotropic non-NMDA receptors, which contribute to the processing of excitatory transmission in the hippocampus (e.g., De Mendonça and Ribeiro, 1993), or by activation of presynaptic high-affinity kainate receptors (Malva et al., 1996). Activation of presynaptic high-affinity kainate receptors has been shown to enhance intracellular free calcium concentration (Malva et al., 1995) and to depress NMDA-mediated excitatory transmission in the rat hippocampus (Chittajallu et al., 1996).

Synaptic transmission in the hippocampus is processed by excitatory pathways, which use glutamate as neurotransmitter, tightly regulated by a network of inhibitory interneurons (Alger, 1991). Thus, to understand the effects of kainate on synaptic transmission in the hippocampus, one has to determine the effects of kainate on the excitatory and inhibitory components. It has been shown that hippocampal glutamate release is inhibited by presynaptic kainate receptors (Chittajallu et al., 1996) and preliminary results suggest that kainate might depress inhibitory synaptic transmission in the hippocampus (Clarke et al., 1996), but it remains to be established whether kainate receptors can directly modulate the release of γ-aminobutyric acid (GABA). The present work was designed to investigate the role of presynaptic high-affinity kainate receptors on the release of GABA from rat hippocampal synaptosomes.

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2. Materials and methods

2.1. Drugs and solutions

Domoic acid, (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), 5-nitro-6,7,8,9-tetrahydrobenzo(*g*)indole-2,3-dione-3-oxime (NS-102), 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were from RBI, (2*S*,4*R*)-4-methylglutamic acid (MGA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Cockson, kainic acid, γ-amino-*n*-butyric acid (GABA), *o*-phthaldialdehyde (OPT), β-mercaptoethanol, aminoxyacetic acid and nipecotic acid were purchased from Sigma, and CGP55845 was supplied by Ciba Geigy. γ-Amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA, specific activity 74.0–88.6 Ci/mmol) was obtained from Amersham. All other reagents were of the highest purity available.

MGA was made up in a 50 mM stock in NaOH (1 M). GYKI 52466 was made up in a 1 mg/ml in methanol, NS-102 was made up in a 5 mM stock in dimethyl sulfoxide (DMSO) and AMPA was made up as a 10 mM stock in DMSO. DPCPX and CGP55845 were made up into a 5 mM and 10 mM stocks in 99% DMSO/1% NaOH (1 M) (v/v). Aqueous dilutions of these stock solutions were made daily.

2.2. Nerve terminal preparation

A synaptosomal fraction from the hippocampus of the rat was prepared as previously described (Cunha et al., 1994). Briefly, male Wistar rats (130–140 g) were decapitated after halothane anesthesia and the brains rapidly removed into ice-cold 0.32 M sucrose solution containing 1 mM EDTA, 1 mg/ml bovine serum albumin, and 5 mM HEPES buffered to pH 7.4 with 1 M NaOH. Halothane anesthesia was used since presynaptic effects of kainate have been consistently observed either without (Malva et al., 1995) or with halothane anesthesia (Chittajallu et al., 1996). The brain was cut longitudinally and the two hippocampi (from three rats) dissected out and homogenized in 10 ml sucrose solution by 4 up-and-down strokes (1500 rpm) in a Teflon/glass apparatus at 4°C. After centrifugation at $3000 \times g$ for 10 min, the supernatant was collected, centrifuged at $14\,000 \times g$ for 12 min and the pellet washed twice with 20 ml of sucrose solution. The washed pellet was resuspended in 1 ml of a solution of 45% (v/v) Percoll in Krebs solution adjusted to 115 mM NaCl with 1 M NaCl and buffered to pH 7.4 with 1 M HEPES/NaOH. The mixture was centrifuged at $14\,000 \times g$ for 2 min and the top layer, which corresponds to the synaptosmal fraction (see Cunha et al., 1992), was removed and washed with 1 ml ice-cold Krebs solution. The pellet (synaptosomal fraction) was resuspended in 1 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution of the following composition (mM): NaCl 125, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, $CaCl_2$ 1.5, $MgSO_4$ 1.2, glucose 10 and aminoxyacetic acid 0.1.

2.3. [^{3}H]GABA release experiments

The synaptosomes were diluted 1:10 in Krebs solution and equilibrated at 37°C for 10 min. From this time onwards, all solutions applied to the synaptosomes were kept at 37°C and continuously gassed with 95% O_2 and 5% CO_2. After the equilibration period, the synaptosomes were loaded with [^{3}H]GABA (1.5 μCi/ml, 1.875 nM), together with 0.006 μM unlabelled GABA for 20 min (loading period). The synaptosomes were then diluted 1:2 with Krebs solution containing 1 μM nipecotic acid. From this time onwards all the solutions contained nipecotic acid (1 μM) to prevent the reuptake of GABA. The synaptosomal suspension was then layered over Whatman GF/C filters into four parallel 90 μl superfusion chambers (adapted from Swinny filter holders, Millipore) through the aid of a roller pump (flow rate: 0.6 ml/min, which was kept constant throughout the experiment). The chamber volume plus dead volume was approximately 0.6 ml. A series of four parallel perfusion chambers was used to enable both control and test conditions to be performed in duplicate from the same batch of synaptosomes. After setting up the synaptosomes, a 30 min washout period was performed before starting sample collection. The effluent was then collected (release period) in 2 min fractions for scintillation counting (500 μl of the effluent to 5 ml Scintran Cocktail T, BDH).

The synaptosomes were stimulated with 20 mM K^+ (isomolar substitution of Na^+ with K^+ in the perfusion buffer) at 4, 22 and 40 min after starting sample collection (S_1, S_2 and S_3). The amount of K^+ used to evoke [^{3}H]GABA and the duration of the pulse was optimized to yield maximal release in a Ca^{2+}-dependent manner. When the effect of any agonist on the release of GABA was investigated, the tested agonist was added to the superfusion medium 6 min before S_2 and remained in the bath up to 34th min. The effect of drugs on the release of [^{3}H]GABA was expressed by alterations of the S_2/S_1 ratio, the ratio S_3/S_1 being used as a control of the recovery of the effect of tested agonists. When we evaluated the modifications of the effect of a tested agonist by an antagonist, this antagonist was applied 15 min before the beginning of the sample collection period and hence was present during S_1, S_2 and S_3. When present during S_1, S_2 and S_3, none of the antagonists significantly altered ($P > 0.05$) the S_2/S_1 or S_3/S_1 ratios as compared with the S_2/S_1 and S_3/S_1 ratios obtained in the absence of antagonists. At the end of the experiments, the filters were removed from the superfusion chambers and analyzed by scintillation counting for determination of tritium retained by the synaptosomes (2 min, error $< 2\%$). Radioactivity was expressed in terms of desintegrations per second per

mg of protein (Bq/mg) in each chamber. The fractional release was expressed in terms of the percentage of total radioactivity present in the preparation at the beginning of the collection of each sample. The amount of radioactivity (expressed as fractional release) released by each pulse of K^+ (S_1 and S_2) was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium release from the total release of tritium obtained upon K^+ stimulation. The basal release was assumed to decline linearly from the 2 min interval before onset of stimulation to the 8th min after onset of stimulation.

2.4. Biochemical assays

Synaptosomal disruption was estimated by comparing lactate dehydrogenase activity, measured by the method of Keiding et al. (1974), in the incubation bath with that found in the synaptosomal pellet upon its solubilization with 2% (v/v) Triton X-100 (Cunha et al., 1992). Synaptosomal disruption during the experiments was small since only $9 \pm 2\%$ of the total lactate dehydrogenase (EC 1.1.1.27) was released. The protein content of the hippocampal synaptosomal fraction was determined according to Spector (1978), using bovine serum albumin as a standard. To verify that both basal and evoked tritium outflow were mainly constituted by [^{3}H]GABA, an aliquot (100 μl) of the synaptosomal superfusate was mixed with 20 μl of 100 μM GABA solution and then derivatized with 100 μl of *o*-phthaldialdehyde solution (27 mg *o*-phthaldialdehyde to 500 μl ethanol with 50 μl β-mercaptoethanol and 4.5 ml borate (0.4 M) buffer pH 9.5). After 2 min, 20 μl of this mixture were analyzed by reverse-phase high-pressure liquid chromatography (Beckman 126 Gold system equipped with a LiChrospher 100 RP-18 column) with fluorescent detection (Jasco, FP-920), as described by Lindroth and Mopper (1979). The HPLC effluent of the peak corresponding to GABA was collected and counted by scintillation counting.

2.5. Statistics

The values are presented as mean ± S.E.M. To test the significance of the effect of an agonist versus control, a paired Student's *t*-test was used. When making comparisons from different set of experiments with control, one-way analysis of variance (ANOVA) was used, followed by Dunnett's test. P values of 0.05 or less were considered to represent significant differences.

3. Results

With the conditions used, the amount of tritium retained by the synaptosomes at the beginning of sample collection was $(1.08 \pm 0.15) \times 10^{10}$ Bq/mg protein ($n = 52$). The basal release of tritium was $(7.2 \pm 0.6) \times 10^6$ Bq/mg protein ($n = 52$) and was mainly ($85 \pm 4\%$, $n = 6$) constituted by [^{3}H]γ-aminobutyric acid ([^{3}H]GABA). Upon stimulation for 2 min with 20 mM K^+ (S_1), $0.25 \pm 0.03\%$ ($n = 52$) of retained tritium was released, which was mainly ($92 \pm 5\%$, $n = 6$) constituted by [^{3}H]GABA. A second stimulation pulse for 2 min with 20 mM K^+ (S_2) also induced the release of tritium (see Fig. 1A), the S_2/S_1 ratio being 0.71 ± 0.02 ($n = 52$). The K^+-induced [^{3}H]GABA release was mostly Ca^{2+}-dependent, since omission of Ca^{2+} from the extracellular medium and addition of 0.5 mM EGTA to the perfusion solution during S_2, decreased the S_2/S_1 ratio by $67 \pm 5\%$ ($n = 6$, $P < 0.05$). As previously shown in striatal synaptosomes (Kirk

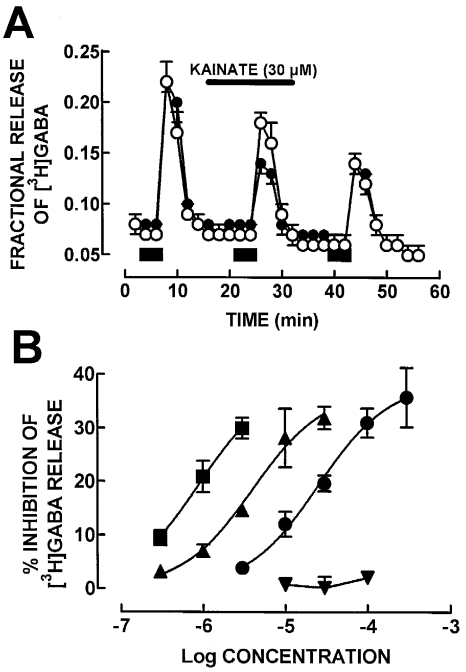

Fig. 1. Time-course inhibition by kainate (30 μM) of [^{3}H]GABA release from hippocampal synaptosomes (A) and concentration-dependent modulation of [^{3}H]GABA release by kainate, domoic acid, methylglutamic acid (MGA) and AMPA (B). The synaptosomes were labeled with [^{3}H]GABA for 20 min, washed, placed in 90 μl chambers and perfused at 0.6 ml/min and stimulated three times with 20 mM K^+ (S_1, S_2 and S_3), as indicated by the lower bars. The evoked tritium outflow was mainly Ca^{2+}-dependent and mostly constituted by GABA (see text) and was assumed to represent exocitotic GABA release. (○) represents [^{3}H]GABA release in the absence of added drugs, whereas (●) represents the effect of kainate (30 μM, present during S_2 as shown in the upper bar in A) on [^{3}H]GABA release. The inhibitory effect of kainate was quantified by modification of the S_2/S_1 ratio which was 0.68 ± 0.02 in control conditions and 0.55 ± 0.03 with 30 μM kainate (four experiments performed in duplicate presented as means ± S.E.M.). In (B), are summarized the concentration-response curves for methylglutamate (■), domoic acid (▲), kainate (●) and AMPA (▼) on the release of [^{3}H]GABA, in experiments performed as in (A). The percentage inhibition by each concentration of the agonists was quantified as the percentage modification of S_2/S_1 versus control experiments performed in parallel for each concentration of each agonist. The results are means ± S.E.M. of 3–4 experiments, except for AMPA (10 and 100 μM) which is $n = 2$. Note that the order of potency of agonists to inhibit [^{3}H]GABA release is methylglutamate > domoic acid > kainate.

and Richardson, 1994), increasing the duration of the K^+ stimulation (to 4 min), or increasing the concentration of K^+ (to 30 mM or 50 mM), maintaining the 2 min period of stimulation, decreased the percentage of Ca^{2+}-dependent [^{3}H]GABA release (data not shown). As previously observed in perfused cortical synaptosomes (Bonanno et al., 1988), the evoked release of [^{3}H]GABA does not appear to be tonically regulated by $GABA_B$ autoreceptors, since CGP55845 (5 μM) did not modify [^{3}H]GABA release ($n = 2$).

As shown in Fig. 1A, when kainate (30 μM) was present during S_2, there was a decrease in the amount of [^{3}H]GABA released, the S_2/S_1 ratio being 0.51 ± 0.04 ($n = 4$). No apparent modification of basal tritium outflow was observed in the presence of kainate (30 μM). In the presence of EGTA (0.5 mM) and without addition of Ca^{2+} to the perfusion solution, kainate (30 μM) increased basal [^{3}H]GABA release by $65 \pm 12\%$ ($n = 3$), but was virtually devoid of effects on evoked [^{3}H]GABA release ($6 \pm 2\%$ inhibition, $n = 3$). Kainate (3–300 μM) produced a concentration-dependent inhibition of [^{3}H]GABA release (Fig. 1B), with a maximal inhibition of $35 \pm 5\%$ and an EC_{50} of 25 μM (95% confidence interval: 18–35 μM, $n = 3$–4). As shown in Fig. 2A, the inhibitory effect of kainate (30 μM) was prevented by the non-NMDA ionotropic receptor antagonist (e.g., Barnes and Henley, 1992), CNQX (10 μM, $n = 4$), and by the kainate receptor-selective antagonist (Johansen et al., 1993), NS-102 (10 μM, $n = 4$). In contrast, the selective non-competitive AMPA receptor antagonist (Donevan and Rogawski, 1993), GYKI 52466 (100 μM, $n = 3$), did not modify the inhibitory effect of kainate (30 μM) (Fig. 2A). The inhibition of GABA release by kainate (30 μM, $16 \pm 4\%$, $n = 3$) in the presence of the adenosine A_1 receptor antagonist, DPCPX (20 nM), was not significantly ($P < 0.05$) different from that obtained in control conditions.

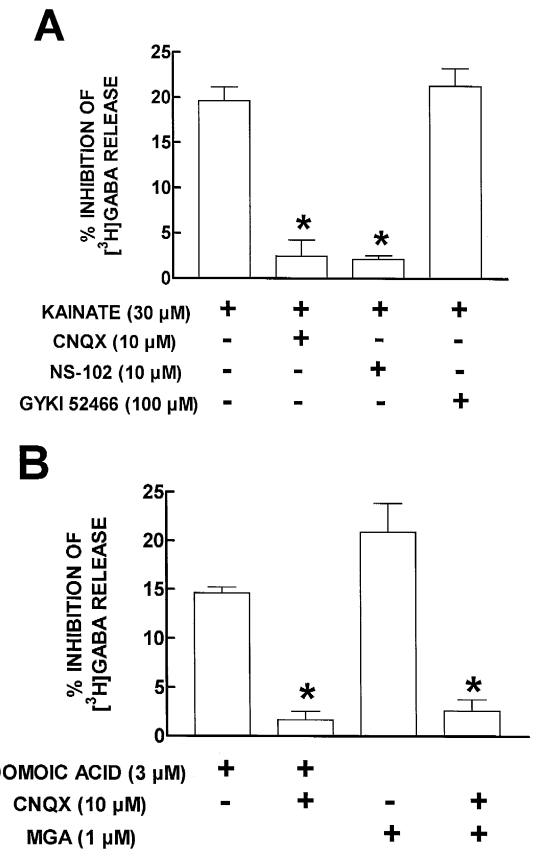

Fig. 2. Pharmacology of the inhibitory effect of kainate (A) and domoic acid and methylglutamate (B) on the evoked release of [^{3}H]GABA from hippocampal synaptosomes. The synaptosomes were stimulated three times with 20 mM K^+ (S_1, S_2 and S_3). The agonists were present during S_2, whereas the antagonists were present from the beginning of perfusion and thus during S_1, S_2 and S_3. The effect of each tested agonist was quantified as the modifications of the S_2/S_1 ratio in the presence of the agonist either versus the S_2/S_1 ratio in the absence of any added drugs or versus the S_2/S_1 ratio in the presence of the tested antagonist, performed in the same experiment with the same batch of synaptosomes. The S_2/S_1 ratio in the presence of each tested antagonist was not statistically ($P > 0.05$) different from the S_2/S_1 ratio obtained in the absence of any added drugs (control S_2/S_1 ratio $= 0.71 \pm 0.02$, $n = 52$). The values are means ± S.E.M. of 3–4 experiments each performed in duplicate. * $P < 0.05$ compared with the effect of the agonist alone.

Domoic acid (0.3–10 μM), which is generally recognized as activating kainic acid-sensitive AMPA-insensitive receptors (e.g., Egebjerg et al., 1991), inhibited GABA release in a concentration-dependent manner, with a maximal inhibition of $32 \pm 2\%$ and an EC_{50} of 4.0 μM (95% confidence interval: 2.3–6.9 μM, $n = 4$) (Fig. 1B). A recently introduced selective agonist of kainate receptors (Gu et al., 1995), (2*S*,4*R*)-4-methylglutamic acid (MGA, 0.3–3 μM), also inhibited [^{3}H]GABA release in a concentration-dependent manner, with a maximal inhibition of $30 \pm 2\%$ and an EC_{50} of 0.90 μM (95% confidence interval: 0.35–2.3 μM, $n = 3$) (Fig. 1B). These kainate receptor agonists produced no apparent modification of basal tritium release. As shown in Fig. 2B, the inhibitory effects of both domoic acid (3 μM) and MGA (1 μM) were prevented by CNQX (10 μM, $n = 3$). AMPA (10–100 μM, $n = 2$–3), which is a poor agonist of kainate receptors (e.g., Bettler and Mulle, 1995), was virtually devoid of effects on [^{3}H]GABA release (Fig. 1B).

4. Discussion

The present results show that presynaptic kainate receptors directly modulate the release of γ-aminobutyric acid (GABA) in whole hippocampal synaptosomes. This was concluded from the concentration-dependent inhibition of [^{3}H]GABA release by kainate and by the kainate receptor agonists domoic acid (Debonnel et al., 1989) and MGA (Gu et al., 1995). The effects of these three agonists were prevented by the AMPA/kainate receptor antagonist, CNQX (Barnes and Henley, 1992). Furthermore, the inhibitory effect of kainate was prevented by the selective kainate receptor antagonist NS-102 (Johansen et al., 1993). It is unlikely that AMPA-preferring receptors could medi-

ate this inhibitory effect on GABA release since AMPA itself was devoid of effects. Moreover, the AMPA-selective antagonist GYKI52466 (Donevan and Rogawski, 1993) did not prevent the inhibitory effect of kainate on GABA release. The effect of kainate was probably not an indirect effect due to the release of other modulators since the perfused synaptosomal preparations eliminate the influence of all so far tested endogenous substances (e.g., Nicholls, 1989), except adenosine (Cunha et al., 1994), the involvement of which was excluded in the present work.

The maximal inhibition by kainate, domoic acid and MGA was only of nearly 30%. Other presynaptic modulatory systems, such as μ-opioid receptor- (Lupica, 1995), or $GABA_B$ receptor-operated systems (Davies et al., 1990) are more efficient to inhibit evoked GABA release in the hippocampus, although they have an heterogenous distribution (Lambert and Wilson, 1993) among different classes of GABAergic neurons (Alger, 1991). An heterogenous distribution of presynaptic kainate receptors has also been observed between hippocampal fields (Malva et al., 1995). It remains to be established if only a subpopulation of GABAergic nerve terminals are endowed with functional kainate receptors or if the kainate receptor system has a lower efficiency.

The EC_{50} values for the inhibitory effects of kainate, domoic acid and MGA are in the low micromolar range, and are higher than the K_i values of these agonists for [^{3}H]kainate binding sites (Egebjerg et al., 1991; Herb et al., 1992; Gu et al., 1995). This difference between EC_{50} values from functional studies and K_d values from binding studies is usually found (see Hollmann and Heinemann, 1994). The EC_{50} values obtained in the present work are also of the same order of magnitude of those reported for the activation of presynaptic kainate receptors on the modulation of intracellular free Ca^{2+} concentration (Malva et al., 1995, 1996), of glutamate release from CA3 synaptosomes (Malva et al., 1996), of glutamate release from whole hippocampal synaptosomes (Chittajallu et al., 1996) and for the activation of homomeric GluR-6 kainate receptors expressed in *Xenopus* oocytes (Egebjerg et al., 1991). The order of potency of agonists to inhibit GABA release (MGA > domoic acid > kainate) also matches that expected for a functionally active kainate receptor (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). These kainate receptors can be constituted by assembled GluR-5/6/7 and KA-1/2 subunits, forming cation-selective channels (e.g., Bettler and Mulle, 1995). Although we could not detect any kainate-induced inhibition of basal [^{3}H]GABA release, to understand an inhibitory effect caused by activation of a cation channel, one has to conceive a progressive depolarization which might first enhance presynaptic excitability but then inactivate Ca^{2+} channels to cause inhibition of neurotransmitter release (for discussion see Chittajallu et al., 1996).

The present results, showing a direct modulation of GABA release by presynaptic kainate receptors in the hippocampus, join previous work (Repressa et al., 1987; Huntley et al., 1993; Debonnel et al., 1989; Gaiarsa et al., 1994; Malva et al., 1995, 1996; Chittajallu et al., 1996) suggesting that the role of kainate receptors in hippocampal synapses might be a neuromodulatory role rather than a direct role in fast excitatory transmission (but see Lerma et al., 1993). Also, the predominant presynaptic location of kainate receptors (see, e.g., Henley, 1995; Repressa et al., 1987) would explain the difficulty in recording kainate receptor-mediated currents (see Bettler and Mulle, 1995), in spite of the heavy labeling of [^{3}H]kainate and of kainate subunit expression in the hippocampus (for reviews, see Hollmann and Heinemann, 1994; Bettler and Mulle, 1995).

The present observations that both kainic and domoic acids directly inhibit GABA release in the hippocampus may contribute to the understanding of the pathological effects of the excitotoxins (Coyle, 1983; Gaiarsa et al., 1994; Teitelbaum et al., 1990). Thus, the proconvulsant and neurotoxic actions of domoic and kainic acids may be understood as the consequence of simultaneous postsynaptic activation of ionotropic non-NMDA receptors (Coyle, 1983; Gaiarsa et al., 1994) and a coordinated increase in glutamate release (Malva et al., 1996; but see Chittajallu et al., 1996) together with a decrease of GABA release. It remains to be established whether MGA also behaves as an excitotoxin.

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