Pertussis toxin prevents presynaptic inhibition by kainate receptors of rat hippocampal [^{3}H]GABA release

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Abstract Kainate receptors are ionotropic receptors, also reported to couple to G_i/G_o proteins, increasing neuronal excitability through disinhibition of neuronal circuits. We directly tested in hippocampal synaptosomes if kainate receptor-mediated inhibition of GABA release involved a metabotropic action. The kainate analogue, domoate (3 μM), inhibited by 24% [^{3}H]GABA-evoked release, an effect reduced by 76% in synaptosomes pre-treated with pertussis toxin. Protein kinase C inhibition attenuated by 82% domoate-induced inhibition of GABA release whereas protein kinase C activation did not change kainate receptor binding. Thus, domoate inhibition of GABA release recruits G_i/G_o proteins and a protein kinase C pathway.

Key words: Kainate receptor; G protein; Protein kinase C; Hippocampus; GABA

1. Introduction

Kainate receptors are a subtype of ionotropic glutamate receptors abundantly expressed in the hippocampus, but their physiological function is only now beginning to emerge [1]. The over-stimulation of kainate receptors leads to epileptiform activity and has proven to be a valuable experimental animal model of temporal lobe epilepsy [2]. One proposed mechanism of kainate-induced epileptogenesis is the disruption of GABAergic transmission [1], which was found in electrophysiological recordings of hippocampal synaptic transmission to involve a metabotropic action of kainate receptors [3]. However, electrophysiological approaches have not unambiguously resolved if kainate receptors presynaptically inhibit GABA release [4] or if dendritic kainate receptors cause a sustained depolarization of GABAergic interneurons indirectly decreasing evoked GABAergic transmission [5]. In such situations where both somatic and presynaptic receptors contribute to a response, electrophysiological approaches have to rely on miniature potential analysis to indirectly study the mechanisms of presynaptic inhibition. However, there are previous examples of presynaptic modulators, such as adenosine, that affect spontaneous and evoked release of neurotransmitters via different mechanisms (discussed in [6]). An alternative is to use an experimental model ideally suited to study the presynaptic modulation of neurotransmitter release, the synaptosomes [7]. Thus, we now directly tested if kainate receptor inhibition of GABA release from hippocampal synaptosomes [8] depends on protein G activation and on protein kinase activities, with care to distinguish if the protein kinases participated on the kainate receptor transducing pathway or were modulating kainate receptor function.

2. Materials and methods

Domoic acid, phorbol-12,13-didecanoate and 4α-phorbol-12,13-didecanoate were from RBI, (*RS*)-α-methyl-4-phosphonophenylglycine (MPPG), (*S*)-α-methyl-4-carboxyphenylglycine (MCPG) and [^{3}H](2*S*, 4*R*)-4-methylglutamate (MGA) were from Tocris Cookson, pertussis toxin, chelerythrine, KN-62, HA-1004, H-89 and PD 98059 were from Calbiochem, γ-amino-*n*-butyric acid (GABA), aminooxyacetic acid, nipecotic acid and 8-bromo-cAMP were from Sigma, CGP 55845 was from Ciba Geigy and [^{3}H]GABA was from Amersham.

[^{3}H]GABA release experiments were performed as previously described [8] using hippocampal synaptosomes, obtained from halothane-anesthetized male Wistar rats (6–8 weeks old), by sucrose/Percoll isopycnic centrifugations. Briefly, synaptosomes were loaded with [^{3}H]GABA (1.5 μCi/ml, 1.875 nM) together with 6 nM unlabelled GABA for 30 min in Krebs solution, gassed with a 95% O_2 and 5% CO_2 mixture, of the following composition: 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, 0.1 mM aminooxoacetic acid, pH 7.4. After loading, the synaptosomes were washed in gassed Krebs solution containing nipecotic acid (1 μM), which was present in all superfusion solutions. The synaptosomes were then layered over Whatman GF/C filters in 90 μl chambers and superfused with gassed Krebs solution with a flow rate of 0.6 ml/min. After 45-min washing, effluent samples were collected in 3-min fractions and 500 μl were used for scintillation counting. The synaptosomes were stimulated twice for 2 min with an isomolar substitution of Na^+ by K^+ (20 mM) 4 min (S_1) and 22 min (S_2) after starting sample collection. Under these conditions, K^+-evoked tritium release is mostly Ca^{2+}-dependent and is mainly due to [^{3}H]GABA release [8], allowing measurement of the evoked [^{3}H]GABA release as evoked tritium release [8,9]. The effect of tested drugs, added to the perfusion medium 6 min before S_2, was evaluated by modification of tritium release in S_2/S_1. When we evaluated the modifications of the effect of domoic acid by other drugs, these drugs were applied 15 min before starting sample collection and hence were present during S_1 and S_2. When present during S_1 and S_2, MPPG plus MCPG, CGP 55845 or any of the tested protein kinase modulators (chelerythrine, HA-1004, H-89, KN-62, PD 98059, phorbol-12,13-didecanoate and 4α-phorbol-12,13-didecanoate) did not significantly alter ($P > 0.05$) the S_2/S_1 as compared to the S_2/S_1 ratio obtained in the absence of drugs (data not shown). When the influence of pertussis toxin pre-treatment on the effect of tested drugs on [^{3}H]GABA release was investigated, the synaptosomes were treated for 2 h with 2 μg/ml of activated pertussis toxin at 37°C (see [10]), before labelling with [^{3}H]GABA. A parallel aliquot of the same synaptosomal batch was always incubated for 2 h with Krebs solution, to control for the influence of the 2 h incubation period on the effect of drugs on [^{3}H]GABA release.

[^{3}H]MGA saturation curves were performed in rat hippocampal

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synaptosomal membranes, as described in [10]. Before preparation of the membranes, the synaptosomes were incubated for 15 min at 37°C without or with either the protein kinase A activator, 8-bromo-cAMP (1 mM), or with the protein kinase C activator, phorbol-12,13-didecanoate (250 nM), as described [11].

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

3. Results and discussion

Since hippocampal kainate receptors can couple to G_i/G_o proteins [10] and pertussis toxin (PTx) attenuates kainate receptor-mediated inhibition of GABAergic transmission [3], we now tested the effect of PTx pre-treatment directly on [^{3}H]GABA release from superfused hippocampal synaptosomes. Pre-treatment of the synaptosomes with PTx (2 μg/ml) for 2 h at 37°C did not change the loading with [^{3}H]GABA and the basal and evoked release of tritium (see Fig. 1). To test the effectiveness of this PTx pre-treatment to inactivate G_i/G_o proteins, we first tested the effect of PTx treatment on the inhibitory effect of $GABA_B$ receptors that are coupled to G_i/G_o proteins in hippocampal GABAergic nerve terminals [12]. The GABA receptor agonist, muscimol (10 μM), inhibited the evoked release of [^{3}H]GABA by 34 ± 4% ($n = 4$) from control synaptosomes (Fig. 1A) and this effect was fully prevented by CGP 55485 (1 μM, present during S_1 and S_2, $n = 3$, Fig. 1B) indicating the involvement of $GABA_B$ receptors. In PTx-treated synaptosomes (Fig. 1B), the inhibitory effect of muscimol (10 μM) was attenuated by 81 ± 6% ($n = 4$) compared to control synaptosomes, confirming that PTx treatment attenuates responses involving G_i/G_o proteins. The kainate receptor agonist, domoate (3 μM) caused a 24 ± 3% inhibition ($n = 4$) of the evoked release of [^{3}H]GABA from control synaptosomes (Fig. 1C), an effect previously shown to be antagonized by the non-NMDA ionotropic glutamate receptor antagonist, CNQX (10 μM) and by the GluR6 antagonist, NS-102 (1 μM), i.e. involving the activation of pharmacologically defined kainate receptors, possibly of the GluR6 subtype [8]. In PTx-treated synaptosomes, domoate only caused a 6 ± 1% inhibition of the evoked release of [^{3}H]GABA ($n = 4$) (Fig. 1D). These results indicate that kainate receptor-mediated inhibition of [^{3}H]GABA-evoked release depends on the presence of functional G_i/G_o proteins. We also excluded the unlikely possibility that activation of $GABA_B$ receptors might mediate domoate inhibition of GABA release, since CGP 55485 (1 μM) did not significantly ($P > 0.05$) modify the inhibition by domoate (3 μM) of [^{3}H]GABA-evoked release (Fig. 1D).

One aspect that remained to be ruled out was the possible direct activation of metabotropic glutamate receptors by domoic acid. The simultaneous presence of the group I and II antagonist, (*S*)-α-methyl-4-carboxyphenylglycine (500 μM),

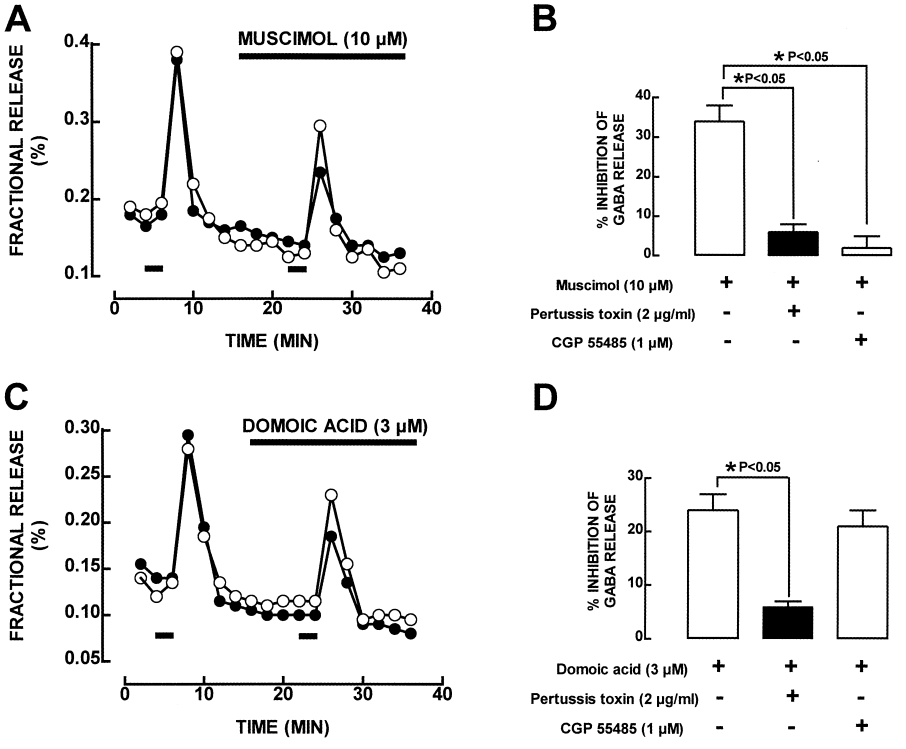

Fig. 1. Inhibition by the $GABA_B$ receptor agonist, muscimol, and by the kainate receptor agonist, domoate, of GABA release from rat hippocampal synaptosomes is sensitive to pertussis toxin. In (A) and (C) the time course of [^{3}H]GABA release is shown. The preparation was challenged with two periods of stimulation with 20 mM K^+ (S_1 and S_2), as indicated by the bars above the abscissa. The open symbols represent tritium release from a control chamber, to which no drug was added, and the filled symbols represent the tritium release of the test chamber, to which either muscimol (10 μM; in A) or domoate (3 μM; in C) was added through the superfusate, as indicated by the upper bar. In (B) and (D) it is shown that pre-treatment of the synaptosomes with activated pertussis toxin (2 μg/ml) attenuated the inhibitory effect of muscimol (10 μM; in B) and of domoate (3 μM; in D). The results are mean ± S.E.M. of four experiments. $*P < 0.05$ versus the inhibitory effect of muscimol (10 μM; in B) or of domoate (3 μM; in C) in control synaptosomes (first column).

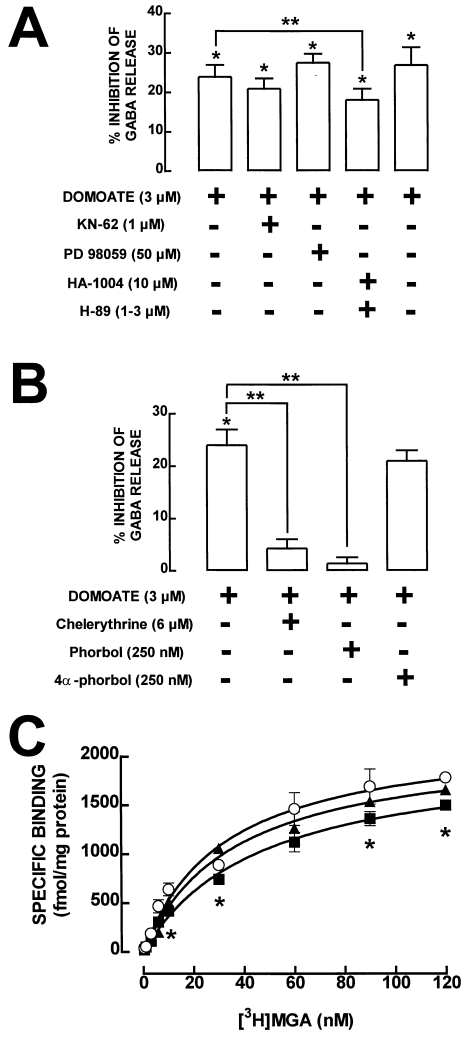

Fig. 2. Ability of inhibitors and activators of protein kinases to affect the kainate receptor-mediated inhibition of GABA evoked release from rat hippocampal nerve terminals (A and B) and [^{3}H]MGA-binding to rat hippocampal synaptosomal membranes (C). In (A) and (B), the protein kinase inhibitors were added 15 min before starting sample collection, i.e. they were present both during S_1 and S_2, whereas domoate was added 6 min before S_2 onwards. The results are mean ± S.E.M. of 3–4 experiments, except for 4α-phorbol-12,13-didecanoate which is $n = 2$. *$P < 0.05$ versus 0%; **$P < 0.05$ versus the inhibitory effect of domoate (3 μM) (first column). In (C) the average saturation curves are shown of [^{3}H]MGA-binding to rat hippocampal synaptosomal membranes upon pre-treatment of the synaptosomes in the absence of drugs (circles) or in the presence of the protein kinase A activator, 8-bromo-cAMP (1 mM, filled squares), or of the protein kinase C activator, phorbol-12,13-didecanoate (250 nM, black and white arrowheads) for 15 min at 37°C. The ordinates represent the specific binding of [^{3}H]MGA on subtraction of the non-specific binding, determined in the presence of 100 μM kainate from total binding. Results are mean ± S.E.M. of three experiments performed in duplicate. The K_D values were 33 nM (95% confidence interval: 22–44 nM) in control conditions, 48 nM (30–66 nM) upon 8-bromo-cAMP pre-treatment and 39 nM (27–60 nM) upon phorbol-12,13-didecanoate pre-treatment. The B_{max} values were 2250 ± 132 fmol/mg protein in control conditions, 2071 ± 53 fmol/mg protein upon 8-bromo-cAMP pre-treatment and 2182 ± 54 fmol/mg protein upon phorbol-12,13-didecanoate pre-treatment.
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and of the group II and III antagonist, (*RS*)-α-methyl-4-phosphonophenylglycine (1 mM), did not attenuate the domoate (3 μM)-induced inhibition of [^{3}H]GABA evoked release, which was 21 ± 2% in the absence and 26 ± 4% in the presence of the metabotropic receptor antagonists ($n = 3$). Thus, it is unlikely that metabotropic glutamate receptor activation may be responsible for the domoic acid induced inhibition of [^{3}H]GABA evoked release.

The triggering of a metabotropic action by kainate receptor activation to inhibit [^{3}H]GABA evoked release was further investigated by testing the effect of protein kinase inhibition on kainate receptor-mediated inhibition of [^{3}H]GABA evoked release. As illustrated in Fig. 2A, the Ca^{2+}-calmodulin kinase II inhibitor KN-62 (1 μM, $n = 3$) or the mitogen-activated protein kinase kinase inhibitor PD 98059 (50 μM, $n = 3$) failed to significantly ($P > 0.05$) modify the domoate (3 μM)-induced inhibition of [^{3}H]GABA evoked release (Fig. 2A), suggesting a lack of direct involvement of these two transducing systems. In contrast, the inhibition by domoate (3 μM) of [^{3}H]GABA-evoked release was attenuated by 82 ± 8% by the protein kinase C inhibitor, chelerythrine (6 μM, $n = 4$) (Fig. 2B). Furthermore, supramaximal activation of hippocampal protein kinase C activity with 250 nM phorbol-12,13-didecanoate (see [11]) also prevented the domoate induced GABA inhibition ($n = 3$) (Fig. 2B), whereas its inactive analogue, 4α-phorbol-12,13-didecanoate (250 nM, $n = 2$) was devoid of effects (Fig. 2B). This confirms the previous suggestion of the involvement of protein kinase C on the presynaptic modulation of GABA release (see [3]). The inhibition by domoate (3 μM) of [^{3}H]GABA evoked release was also inhibited by 26 ± 4% by the protein kinase A inhibitor HA-1004 (10 μM, $n = 4$) (Fig. 2A). However, the more selective protein kinase A inhibitor, H-89 (1 μM, $n = 2$, or 3 μM, $n = 2$) did not modify the domoate (3 μM)-induced inhibition of GABA release (Fig. 2A). This suggests that the protein kinase A pathway is not recruited by kainate receptors, as reported by others [3], and the effect of HA-1004 might be due to its ability to inhibit protein kinase C due to its low protein kinase A versus C selectivity [13].

Kainate receptor function has previously been shown to be controlled by protein kinases, namely by protein kinase A (e.g. [14]). Therefore, it is important to distinguish if protein kinases are triggered by kainate receptor activation or if protein kinases are controlling kainate receptor function. To investigate this issue, we tested the ability of the membrane-permeable protein kinase A activator, 8-bromo-cAMP (1 mM) and of the protein kinase C activator phorbol-12,13-didecanoate (250 nM) to modify binding to kainate receptors. The pre-treatment of hippocampal synaptosomes for 15 min with the protein kinase C activator failed to significantly ($P > 0.05$) modify [^{3}H]MGA saturation curve to membranes prepared from these synaptosomes when compared to control (Fig. 2B), which contrasts with the reduction in K_D caused by PTx [10]. However, activation of protein kinase A lead to a reduction in the binding of the higher concentrations of [^{3}H]MGA (Fig. 2B), although the changes in K_D and B_{MAX} did not reach statistical significance ($P > 0.05$). Thus, the present results support the view that protein kinase C may be part of the transducing pathway of kainate receptor-mediated inhibition of GABA release rather than directly regulating kainate receptor function. In contrast, protein kinase A is not part of the transducing system operated by kainate recep-

tors to inhibit GABA release but may directly regulate kainate receptor function in the hippocampus.

The present results demonstrate that kainate receptor activation requires functional G_i/G_o proteins to inhibit GABA release from hippocampal nerve terminals, confirming previous electrophysiological data indicating a PTx-sensitive kainate receptor inhibition of GABAergic transmission in hippocampal CA1 area [3]. The qualitative similarity of PTx sensitivity of this direct presynaptic effect of kainate receptor activation on GABA release and on GABAergic transmission is suggestive of a main presynaptic locus of kainate receptor action in the control of GABAergic function in the hippocampus. Like presynaptic kainate receptors, postsynaptic AMPA receptors can also signal through G proteins (e.g. [15]). However, it is still not clear if these ionotropic glutamate receptors directly couple to G_i/G_o proteins or more likely indirectly couple to G proteins via ancillary proteins (e.g. [16]). Also, the metabolic route operated by presynaptic kainate receptors downstream of G_i/G_o proteins remains to be determined, but appears to be different from that triggered by postsynaptic AMPA receptors (see [15]).

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