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Presynaptic kainate receptor-mediated facilitation of glutamate release involves Ca^{2+}–calmodulin and PKA in cerebrocortical synaptosomes

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ABSTRACT

We have explored the mechanisms involved in the facilitation of glutamate release mediated by the activation of kainate receptors (KARs) in the cortex using isolated nerve terminals (synaptosomes). Kainate (KA) produced an increase on glutamate release at 100 μM. The effect of KA was antagonized by NBQX (with AMPA receptors blocked by GYKI53655). This facilitation was suppressed by the inhibition of PKA activation by Rp-Br-cAMP and H-89. Moreover, the facilitation of glutamate release mediated by KAR requires the mobilization of intrasynaptosomal Ca^{2+} stores and the formation of a Ca^{2+}–calmodulin complex. We conclude that KARs present on presynaptic terminals in the neocortex mediate the facilitation of glutamate release through a mechanism involving an increase in cytosolic Ca^{2+} to activate a Ca^{2+}–calmodulin–AC/cAMP/PKA signaling cascade.

1. Introduction

As the major excitatory neurotransmitter in the mammalian central nervous system (CNS), glutamate supports normal synaptic transmission, as well as sustaining learning and memory processes, manifest experimentally as long-term potentiation (LTP) and long-term depression (LTD) of synapses [1]. Glutamatergic neurotransmission also plays a developmental role in synaptogenesis and neuronal maturation [1]. The ionotropic glutamate receptor family has three members, viz. NMDA-, AMPA- and Kainate-type receptors [2], all of which are homo- or hetero-meric tetramers composed from cognate sets of diverse subunits. Kainate receptors (KARs) are constructed from GluK1, GluK2, GluK3, GluK4 and GluK5 subunits, with the former three able to form low-affinity homotetramers, with heterotetrameric assemblies including GluK4 and GluK5 imparting higher agonist affinities on the receptor complexes [3].

Found ubiquitously distributed in the CNS, KARs were in the first instance described as being postsynaptic, being located in the principal cells and interneurons of the hippocampus, the lateral amygdala, dorsal root ganglia, bipolar cells of the retina, cerebral cortex, globus pallidus and cerebellum [3–5]. Persuasive evidence subsequently identified the presynaptic terminal localization of KARs, whereby they modulate neurotransmitter release [3–7]. KARs have been implicated in the modulation of both glutamate and GABA release [3–7]. At some glutamatergic synapses, KAR activation mediates a biphasic effect. Thus, while low concentrations of the agonist kainate (KA) produce an increase in glutamate release, high concentrations effect a decrease in glutamate release [4,5]. The exact mechanism by which KARs produce the former facilitation of glutamate release remains to be fully elucidated. Indeed, the precise location of receptors that are responsible for this facilitation is yet to be demonstrated.

Here, we have examined the effect of KA on glutamate release from isolated cerebrocortical nerve terminals (synaptosomes) prepared using a well established procedure [8–11]. In this preparation, any confounding postsynaptic effects of KA on glutamate release are obviated by the minimal presence of functional postsynaptic elements [8–12]. We found that the facilitation of glutamate release showed a major sensitivity to suppression of cAMP-mediated activation of protein kinase A (PKA), depletion of intrasynaptosomal Ca^{2+} stores and inhibition of calmodulin. The data implicate Ca^{2+}–calmodulin stimulation of adenylyl cyclase (AC)

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and downstream activation of PKA in the KAR-mediated facilitation of glutamate release.

2. Methods

2.1. Animals

Synaptosomes were obtained from male, adult Sprague–Dawley rats (150–200 g). Experiments were carried out according to the Home Office Animals (Scientific Procedures) Act of 1996.

2.2. Preparation of synaptosomes

Synaptosomes were prepared from the cerebral cortex as described previously [8]. The final synaptosomal fraction was resuspended in HEPES-buffered incubation medium (HBM) containing (mM): 140 NaCl, 5 KCl, 5 $NaHCO_3$, 1 $MgCl_2{\cdot}6H_2O$, 1.2 Na_2HPO_4, 10 glucose, 20 HEPES (pH 7.4). Protein concentration was then determined using a Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.5 mg protein. Synaptosomal pellets were stored on ice and used within 1–2 h. We have shown this well established preparation to be enriched in synapsin I, a exclusively presynaptic marker [12]. Further the robust metabolic competence and ability of percoll purified synaptosomes to release neurotransmitters [13,14], has made this preparation a persuasive model for elucidating presynaptic receptor function [15].

2.3. Glutamate release assay

Glutamate release was assayed by on-line fluorometry [16]. Pelleted synaptosomes were resuspended at a protein concentration of 0.5 mg/ml in HBM containing 16 μM bovine serum albumin (BSA) and incubated in a stirred and thermostatted cuvette at 37 °C in a Perkin–Elmer LS-3B spectrofluorimeter. $NADP^+$ (1 mM), glutamate dehydrogenase (50 units/ml) and $CaCl_2$ (1 mM) were added after 3 min. After a further 10 min of incubation, 1 mM 4-aminopyridine (4-AP) was added to stimulate glutamate release. The oxidative deamination of released glutamate, leading to the reduction of $NADP^+$, was monitored by measuring NADPH fluorescence at excitation and emission wavelengths of 340 and 460 nm, respectively. Data were accumulated at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence change produced by the standard addition was used to calculate the released glutamate as nanomoles glutamate per milligram synaptosomal protein. Release traces are shifted vertically to align the point of depolarization as zero release. Release values quoted in the text are levels attained at "steady-state" after 4 min of depolarization (nmol/mg protein/4 min).

2.4. Data analysis

Data are presented as mean ± S.E.M. Each *n* indicates the number of individual synaptosome preparations used; each preparation was derived from a single animal. Significance was assessed at $P < 0.05$, using the Mann–Whitney U test.

2.5. Compounds

Kainate, salts and general reagents were purchased from Sigma (St. Louis, MO); GYKI 53655, CNQX, NBQX, D-AP5, thapsigargin, ryanodine, CMZ and W-7 were obtained from Tocris (Bristol, UK).

3. Results

3.1. Kainate receptor activation increases glutamate release in cerebrocortical synaptosomes

Using an on-line enzymatic assay for measuring glutamate, we observed a KA-mediated facilitation of glutamate release from cerebral cortex nerve terminals (synaptosomes) as described previously [16] (Fig. 1A). We applied KA to cerebrocortical synaptosomes in the presence of 30 μM GYKI 53655 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine, a non-competitive AMPA/KA receptors antagonist which at, 30 μM, is selective for AMPA receptor and does not affect KAR activity [17,18]) or 100 μM SYM2206 (±4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7methylenedioxyphtalazine, another non-competitive AMPA receptor antagonist [19]), to prevent the activation of AMPA receptors by KA. Under these conditions, the application of 100 μM KA produced a clear and statistically significant facilitation of glutamate release (37 ± 6%, $n = 12$, Fig. 1A and B)

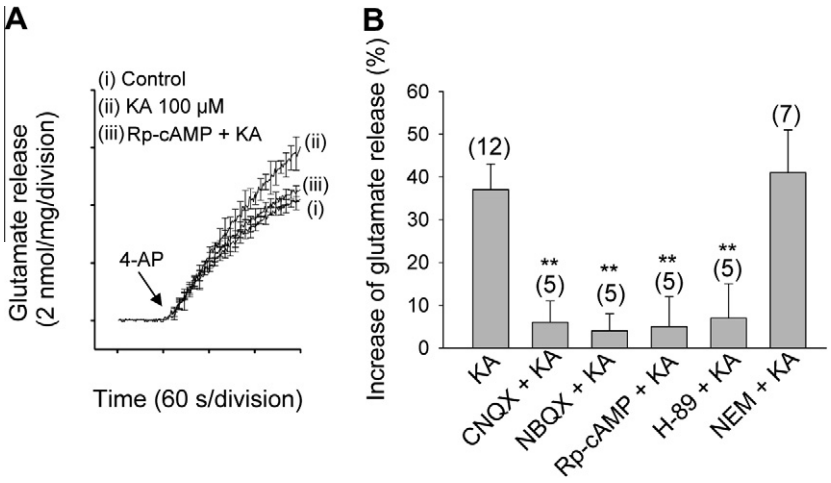

Fig. 1. Kainate-induced facilitation of 4-AP-evoked glutamate release in cerebrocortical synaptosomes requires PKA but not G-protein action. (A) Glutamate release in the absence (i) and presence (ii) of 100 μM KA (added 1 min before the addition of 4-AP), (iii) increase in glutamate release is prevented in the presence of PKA inhibitor Rp-cAMP. (B) Quantification of modulation using release levels achieved 4 min post 4-AP. Increase of glutamate release by KA is prevented in the presence of CNQX, NBQX, Rp-Br-CAMP and H-89 but not in the presence of NEM. The numbers in parentheses indicate the number of experiments using independent synaptosomal preparations. Results are the mean ± S.E.M. (**$P < 0.01$, Mann–Whitney U test).

evoked by 1 mM 4-AP. We next re-examined the observed KA-induced facilitation in the presence of the AMPA/kainate receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione [20], 100 μM) and NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [*f*]quinoxaline-7-sulfonamide, [21], 10 μM). In our incubation conditions, given that we routinely blocked AMPA receptors with GYKI53655 or SYM2206, CNQX and NBQX are effectively specific kainate receptor antagonists. In the presence of CNQX, the facilitatory effect of KA was blocked (6 ± 5% increase, $n = 5$) as well as in the presence of NBQX (4 ± 4%, $n = 5$). The abrogation of the effect of KA in the presence of CNQX is not attributable to the inhibitor targeting NMDA receptors [22] since the NMDA receptor antagonist, D-AP5 (D(−)-2-amino-5-phosphonovaleric acid (50 μM), had no effect on the facilitation of glutamate release (35 ± 7%, $n = 5$) produced by KA. These results indicate (as previously described in [15]) that the facilitation of glutamate release that we observe is mediated by the activation of a presynaptic KAR (Fig. 1A and B). These data suggest that the selective activation of a presynaptic glutamate receptor of the kainate type produces a facilitation of glutamate release in cerebrocortical nerve terminals.

3.2. Kainate-induced facilitation of glutamate release involves the cAMP cascade in cerebral cortex synaptosomes

The mechanism underlying the facilitation of glutamate release by KA receptors remains to be elucidated in cerebrocortical synaptosomes. Having confirmed the selectivity of the action of KA on glutamate release, we further explored the mechanism(s) underlying the effect. We previously described in hippocampal synaptosomes, that KAR-mediated facilitation involves signaling instigated by cAMP [23]. We therefore analyzed the effect of inhibiting the activation of cAMP-dependent protein kinase A (PKA) on

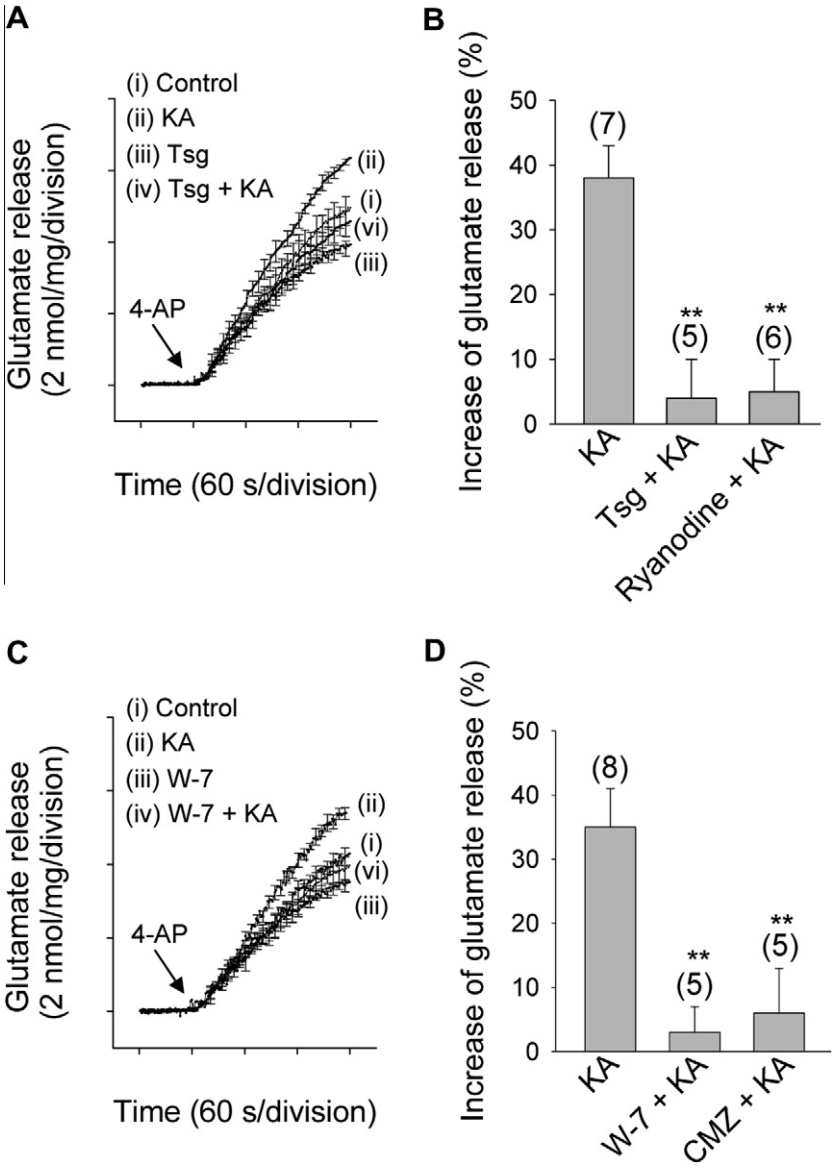

Fig. 2. Facilitation of 4-AP-evoked glutamate release mediated by KAR activation requires release of Ca^{2+} from intrasynaptosomal stores and the formation of a Ca^{2+}/calmodulin complex in cerebrocortical synaptosomes. (A) Glutamate release under control conditions (i) and in the presence of 100 μM KA (ii), thapsigargin (Tsg) (iii), and Tsg + KA (iv). (B) Quantification of modulation observed in A and in the presence of ryanodine using release levels achieved 4 min post 4-AP. The effect of KA is prevented in Tsg and ryanodine-treated synaptosomes. (C) Glutamate release under control conditions (i) and in the presence of 100 μM KA (ii), W-7 (iii), and W-7 + KA (iv). (D) Quantification of modulation observed in A and in the presence of CMZ using release levels achieved 4 min post 4-AP. The effect of KA is prevented in W-7 and CMZ-treated synaptosomes. The number of experiments is indicated in parentheses at the top of each bar. Results are expressed as means ± S.E.M. (**$P < 0.01$, Mann–Whitney U test).

KA-mediated facilitation of glutamate release from cerebrocortical synaptosomes. For this purpose, we used the selective and inactive cAMP analogue, Rp-Br-cAMP (bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer [24], 100 μM) and the inhibitor H-89 (*N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide [25], 2 μM) to effect the inhibition of cAMP-dependent activation of PKA in nerve terminals. In the presence of Rp-Br-cAMP and H-89, subsequent application of 100 μM KA did not facilitate glutamate release (5 ± 7%, $n = 5$ and 7 ± 8%, $n = 6$, respectively) versus the 36 ± 6% ($n = 6$) facilitation of glutamate release obained without Rp-Br-cAMP or H-89. These results indicate that inhibition of PKA activation abrogates the regulatory action of KA (Fig. 1A and B). Notably however, although some of the effects of KARs have been attributed to a metabotropic mechanism involving G-proteins [4–5,26], we observed no effect of general G-protein inhibition by NEM (*N*-ethylmaleimide, alkylating agent that effects inactivation of G proteins [27], 2 μM, 41 ± 10%, $n = 7$, versus 40 ± 7%, $n = 5$, Fig. 1B) on the facilitation of glutamate release mediated by KAR activation. The instigation of endogenous cAMP production implicated by the foregoing data must therefore arise from a G-protein-independent activation of adenylyl cyclase (AC) in synaptosomes.

3.3. Facilitation of glutamate release mediated by presynaptic KAR activation requires an increase of Ca^{2+} in the cytosol and involves Ca^{2+}–calmodulin in cerebrocortical synaptosomes

In the hippocampal slice studies, KAR-mediated facilitation of glutamate release has been suggested to involve Ca^{2+} increases in the cytosol, potentially through mobilization of intracellular Ca^{2+} stores [27–30]. To determine whether intrasynaptosomal Ca^{2+} stores underpin or support the KAR-mediated facilitation of glutamate release, we performed experiments in cerebrocortical synaptosomes treated with thapsigargin (2 μM), a smooth endoplasmic reticulum Ca^{2+}-ATPase inhibitor known to deplete Ca^{2+} from intracellular stores [28,31]. In this condition, KA produced no facilitation of glutamate release in cortical synaptosomes (4 ± 6%, $n = 5$, Fig. 2A and B). The intracellular release of Ca^{2+} observed could be triggered either by inositol trisphosphate (IP_3), or via Ca^{2+} induced Ca^{2+} release. We therefore tested the latter by looking at the ability of ryanodine (10 μM) to block KAR-mediated facilitation. Ryanodine, which selectively inhibits Ca^{2+} induced Ca^{2+} release [32], prevented KARs mediated facilitation of glutamate release (5 ± 5%, $n = 6$, Fig. 2B). These results clearly indicate that an increase in cytosolic Ca^{2+}, involving intrasynaptosomal Ca^{2+} stores, is obligatory for the facilitation of glutamate release mediated by KAR-activation.

From foregoing results, it is clear that an increase in Ca^{2+} concentration in the cytosol after KAR activation is necessary for the mediation of the facilitation of glutamate release produced by KA, where the activation of an AC/cAMP/PKA pathway evidently underpins the regulation. Given that we have also observed that KAR-mediated facilitation does not involve canonical G-protein activation, the question remains, how could AC then be activated? Knockout experiments have established an important role for Ca^{2+}–calmodulin stimulated adenylyl cyclases (i.e., AC1 and AC8) in the hippocampus [33–35]. This raises the prospect that activation of the aforementioned AC(s) in cerebrocortical nerve terminals may occur through the increase in Ca^{2+} activating calmodulin. We tested for this possibility in cerebrocortical synaptosomes by examining the effect of inhibiting Ca^{2+}–calmodulin by using the calmodulin antagonist W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide [36]. In the presence of W-7 (25 μM), the facilitatory effect of KA on glutamate release was abolished (3 ± 4%, $n = 5$ increase, Fig. 2C and D). W-7 had no statistically significant effect on glutamate release under control conditions (in the absence of KA, −10 ± 5%, $n = 5$, Fig. 2C). We also performed the experiment in the presence of an alternative calmodulin antagonist calmidazolium, CMZ (1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-3-(2,4-dichlorobenzyloxy) phenethyl] imidazolinium chloride [37], 1 μM). As with W-7, in synaptosomes treated with CMZ, KA-mediated facilitation of glutamate release was abolished (6 ± 7%, $n = 5$, Fig. 2B). These results indicate that a presynaptic Ca^{2+}–calmodulin complex is necessary for the activation of the increase of glutamate release after KA application and may form the basis of the mandatory upstream AC activation shown.

4. Discussion

Using biochemical studies in cerebrocortical nerve terminals, our results show that the activation of presynaptic KARs produces an increase of glutamate release and that these receptors are coupled to a cascade involving Ca^{2+}-calmoduin/AC/cAMP/PKA activity (Fig. 3). As synaptosomes are devoid of functional postsynaptic elements, the experiments are demonstrative of presynaptic modulation. Importantly, the observed effect of KA could be attributed specifically to the activation of KARs, as synaptosomes were incubated in the presence of the AMPA receptor antagonist GYKI53655, and in continued presence of the latter, the clear facilitation of glutamate release by KA addition was completely blocked by treatment with CNQX or NBQX (implying no other ionotropic glutamate receptors are involved). Furthermore, the increase in glutamate release produced by KA was eliminated when synaptosomes were incubated with the inhibitors Rp-Br-cAMP and H-89, confirming the involvement of PKA in the KAR-mediated facilitation. The pretreatment of synaptosomes with thapsigargin or ryanodine abolished the KA-mediated effect. This demonstrates that increases in cytosolic Ca^{2+} concentrations involving intrasynaptosomal Ca^{2+} store mobilization is necessary for the KAR-mediated increase of glutamate release observed.

Our previous and current results using synaptosomes have indicated that the KAR-mediated action on glutamate release is not mediated by G-proteins (unaffected by NEM treatment). To gain further insight into the mechanism of KARs-mediated increase of glutamate release, and to determine how the activation of AC might occur in the absence of G-protein activation, we studied the mechanistic details of the involvement of increased cytosolic Ca^{2+} in the regulation effected by KARs. Using the calmodulin antagonists, W-7 and CMZ, we evaluated the participation of calmodulin in this action of KA. Although CMZ has been reported to

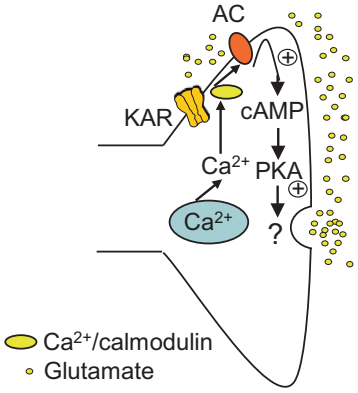

Fig. 3. Mechanism of KAR-mediated facilitation of glutamate release at cerebrocortical synaptosomes. Schematic diagram showing that KA facilitates glutamate release by activating KARs and inducing Ca^{2+} release from intraterminal stores. The increase of cytosolic [Ca^{2+}] mediates the formation of a Ca^{2+}–calmodulin complex which activates AC and thereby PKA subsequently.

have some calmodulin-independent actions, these have been largely noted in tissues other than the brain [37,38]. In the brain at a concentration of 1 μM, the primary target for CMZ is calmodulin. In the presence of either calmodulin inhibitor, the KA-mediated modulation was abolished. Evidently therefore, any increased cytosolic Ca^{2+} initiated by KAR activation requires a Ca^{2+}–calmodulin complex to effect the modulation of glutamate release, putatively instigated by a Ca^{2+}–calmodulin-sensitive AC operating in a AC/cAMP/PKA signaling cascade. Of all identified ACs, type 1 and 8 (AC1 and AC8) are the major Ca^{2+} stimulated ACs in the central nervous system [35,39]. In fact, double knock-out mice, lacking both AC1 and AC8, do not show Ca^{2+}-stimulated elevation of cAMP [40]. The proposed formation of the Ca^{2+}–calmodulin complex following KAR activation described herein may indeed therefore activate AC1 and/or AC8 to initiate the KA mediated facilitation of glutamate release.

In conclusion, our data show that the activation of presynaptic KARs by KA in cereborcortical syanaptosomes results in the facilitation of glutamate release by a mechanism that involves the triggering of release of Ca^{2+} from intrasynaptosomal stores. The Ca^{2+} putatively binds to calmodulin to form a Ca^{2+}–calmodulin complex, which then likely activates AC1 or AC8 to produce an increase in cAMP levels and an activation of PKA, thereby resulting in the facilitation of glutamate release.

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