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Cyclothiazide unmasks AMPA-evoked stimulation of [^{3}H]-L-glutamate release from rat hippocampal synaptosomes

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The effect of α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) on Ca^{2+}-sensitive, tetrodotoxin (TTX)-insensitive K^+-stimulated [^{3}H]-L-glutamate release from rat hippocampal synaptosomes was determined. AMPA in the presence, but not in the absence of cyclothiazide, a drug which blocks AMPA receptor desensitization, elicited a dose-dependent increase in K^+-stimulated [^{3}H]-L-glutamate release but had no effect on basal release. The AMPA/cyclothiazide stimulation was blocked by CNQX and by GYKI 52466, an antagonist at the cyclothiazide site. These results indicate that AMPA receptors are present on presynaptic terminals and suggest that they may play a role in the regulation of neurotransmitter release.

Keywords: AMPA; GYKI; cyclothiazide; CNQX; synaptic plasticity; glutamate release; autoreceptors

Introduction Ionotropic glutamate receptors can be subdivided into N-methyl-D-aspartate (NMDA) and non-NMDA (kainate and α-amino-3-hydroxy-5-methylisoxazole-propionate (AMPA)) subtypes and, to date, at least seven separate cDNAs encoding non-NMDA receptor subunits have been cloned and characterized. Among these GluR1-GluR4 are proposed as the constituent subunits of functional AMPA receptors (Wenthold *et al.*, 1992).

AMPA receptors are important in a range of physiological (Barnes & Henley, 1992) and pathophysiological (Choi, 1992) processes including a long-term potentiation (LTP) in the rat hippocampus. LTP is of widespread interest since it has been proposed as an underlying molecular mechanism for learning and memory (Bliss & Collingridge, 1993). Nonetheless, while the involvement of postsynaptic AMPA receptors in LTP has been demonstrated (Bliss & Collingridge, 1993) the existence of presynaptic AMPA receptors has not been established. Here we present evidence for funtionally relevant AMPA receptors on the presynaptic terminals in the rat hippocampus which regulate the release of neurotransmitter.

Methods The hippocampil were dissected from two female Wistar rats (100-170 g) per experiment and synaptosomes were prepared as described previously (McMahon *et al.*, 1989). The synaptosomes were incubated in pre-gassed (O_2/CO_2; 95/5%) Krebs buffer (mM; NaCl 118.0, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.0) plus [^{3}H]-L-glutamate (25 nM; 50 Ci mmol^{-1}) at 37°C for 7 min. The uptake reaction was terminated by two washes in 10 volumes of ice-cold Krebs buffer and the washed [^{3}H]-L-glutamate-loaded synaptosomes were resuspended to 5 ml in fresh ice-cold Krebs.

Aliquots (0.1 ml) were added to 0.9 ml of either pre-warmed Krebs buffer, high K^+-Krebs buffer (containing an additional 20 mM KCl which gives approximately half-maximal stimulation of release) or Krebs buffer containing 50 μM 4-aminopyridine (4-AP) in the absence (control) or presence of drugs and incubated at 37°C for 2 min. Released [^{3}H]-L-glutamate was separated from synaptosomal [^{3}H]-L-glutamate by rapid filtration through GF/B filters followed by a 1 ml wash with ice-cold Krebs buffer. The data are expressed as percentage of control stimulated release as defined by the difference between the basal and K^+-stimulated [^{3}H]-L-glutamate release in the absence of drugs (100%).

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Greater than 75% of the K^+-stimulated [^{3}H]-L-glutamate release was Ca^{2+}-dependent and K^+-stimulated release was not blocked by the voltage-sensitive Na^+ channel blocker tetrodotoxin (100 nM) indicating that release was from synaptosomes comprising presynaptic terminals. Analysis by high performance liquid chromatography (h.p.l.c.) confirmed that the tritium released was in the form of [^{3}H]-L-glutamate rather than any ^{3}H-labelled metabolic product (data not shown).

Results The pharmacological integrity of the synaptomes was confirmed using the previously characterized ability of carbachol to inhibit endogenous and exogenously applied neurotransmitter release (Marchi *et al.*, 1989) Consistent with those previous observations, in our assay system carbachol (100 μM) reduced K^+-stimulated release by 40% and that inhibition was completely inhibited by 0.1 μM atropine (Figure 1).

AMPA alone had no effect on stimulated release but in the presence of cyclothiazide AMPA potentiated K^+-stimulated but not basal [^{3}H]-L-glutamate release (Figure 2a) in a concentration-dependent manner (Figure 2b). The maximum

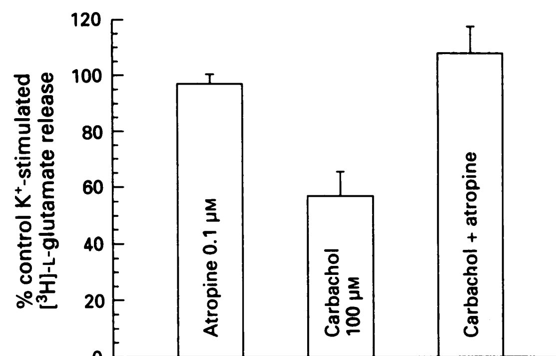
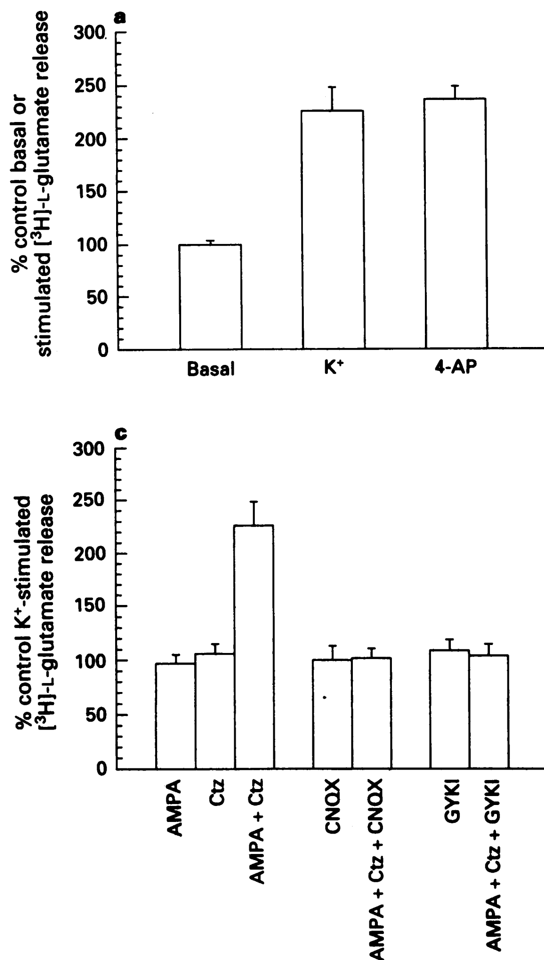
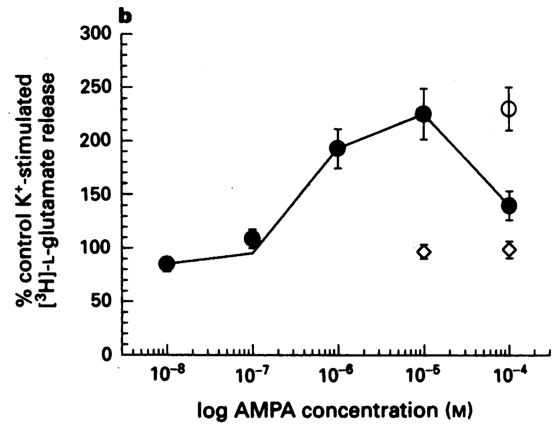


Figure 1 Carbachol inhibition of [^{3}H]-L-glutamate release. Effects of the inhibitory mucarinic agonist carbachol (100 μM) and specific antagonist atropine (0.1 μM) on 25 mM K^+-stimulated [^{3}H]-L-glutamate release. The data are the mean ± s.e.mean of 6-8 separate experiments each performed in triplicate. In normal Krebs buffer 100 μl aliquots of synaptosomes prepared as described in the Methods released ~10,000 d.p.m. into the supernatant during the 2 min time course of the experiment. In the presence, but not in the absence of $CaCl_2$, high K^+-Krebs resulted in 13,000-15,000 d.p.m. and 50 μM 4-AP resulted in ~15,000 d.p.m. of [^{3}H]-L-glutamate being released.





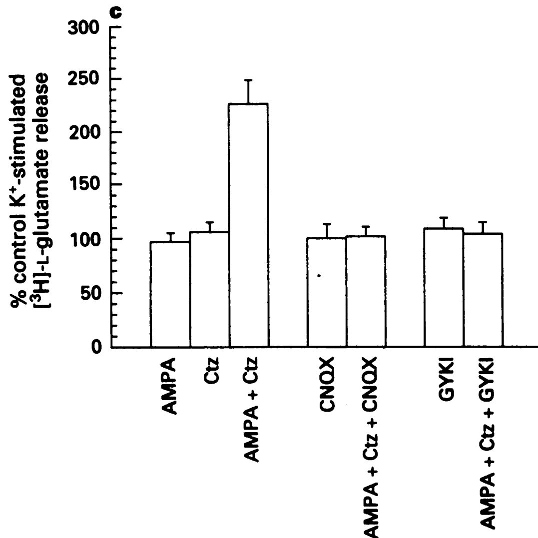


Figure 2 AMPA potentiation of [^{3}H]-L-glutamate release. (a) Effects of 10 μM AMPA + 10 μM cyclothiazide on basal, 25 mM K^+-stimulated and 50 μM 4-AP-stimulated [^{3}H]-L-glutamate release. The data are the mean ± s.e.mean of 3–8 separate experiments each performed in triplicate. (b) Concentration-response curve for AMPA-evoked increase in K^+-stimulated [^{3}H]-L-glutamate release in the presence of 10 μM cyclothiazide or 100 μM cyclothiazide. The data are the means ± s.e.mean of 8–12 separate experiments each using synaptosomes prepared from 4 hippocampi: (●) represent increasing concentrations of AMPA in the presence of 10 μM cyclothiazide. The bell-shaped dose-response curve does not occur with 100 μM cyclothiazide (○); (◇) show the lack of effect of 10 μM and 100 μM AMPA in the absence of cyclothiazide (mean ± s.e.mean of 4 separate experiments each performed in triplicate). (c) Blockade of 10 μM AMPA plus 10 μM cyclothiazide-evoked increases in K^+-stimulated [^{3}H]-L-glutamate release by CNQX (100 μM) and GYKI 52466 (10 μM). Means ± s.e.mean of 8–12 separate experiments each performed in triplicate.

increase in K^+-stimulated [^{3}H]-L-glutamate release was ~220% with an EC_{50} value of 0.3 μM. The AMPA/cyclothiazide response was blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and the 2,4-benzodiazepine GYKI 52466 (1-(4'amino-phenyl)-3-acethyl-4-methyl-3,4-dihydro-7, 8-methylenedioxy-5H-2,3-benzodiazepine) (Figure 2c).

Discussion The bell-shaped dose-response for AMPA (Figure 2b) can be attributed to 10 μM cyclothiazide failing to block 100 μM AMPA-evoked receptor desensitization since the decrease does not occur with 100 μM cyclothiazide. Consistent with the results presented here, cyclothiazide potentiation of AMPA-induced [^{3}H]-noradrenaline release from rat hippocampal slices has also been observed recently but antagonism by GYKI 52466 was not reported (Desai *et al.*, 1994).

The AMPA/cyclothiazide reponse was inhibited by CNQX and GYKI 52466. CNQX is a well characterized competitive antagonist at the AMPA binding site (Barnes & Henley, 1992) whereas GYKI 52466 is a highly selective non-competitive antagonist of AMPA-evoked responses (Donevan & Rogawski, 1993) and excitotoxicity (Zorumski *et al.*, 1993) in rat CNS. The mechanism of inhibition by GYKI 52466 has been proposed as promoting the rate of desensitization of AMPA receptors by interaction at the cyclothiazide site. Thus GYKI 52466 is believed to be a competitive antagonist at the same allosteric site at which cyclothiazide is an agonist (Zorumski *et al.*, 1993).

The results presented here are relevant to the debate over possible presynaptic mechanisms for the induction and maintenance of LTP. While the molecular mechanisms regulating neurotransmitter release *in vivo* remain to be determined, our data indicate that activation of presynaptic AMPA receptors, under conditions where desensitization is prevented, can increase release. Furthermore, we demonstrate that the recently available compounds cyclothiazide and GYKI 52466 and their subsequent derivatives will provide invaluable tools to study the properties of pre- and post-synaptic AMPA receptors.

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