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Cyclothiazide and AMPA receptor desensitization: analyses from studies of AMPA-induced release of [^{3}H]-noradrenaline from hippocampal slices

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1 Responses in brain produced by the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of ionotropic receptor for L-glutamate are often rapidly desensitizing. AMPA-induced desensitization and its characteristics, and the potentiating effect of cyclothiazide were investigated *in vitro* by analysing AMPA-induced release of [^{3}H]-noradrenaline from prisms of rat hippocampus.

2 AMPA (1–1000 μM) stimulated the release of [^{3}H]-noradrenaline in a concentration-dependent manner that was both calcium-dependent and tetrodotoxin-sensitive, and attenuated by the AMPA-selective antagonists, NBQX (1 and 10 μM), LY 293558 (1 and 10 μM) and GYKI 52466 (10 and 30 μM).

3 By use of an experimental procedure with consecutive applications of AMPA (100 μM, 28 min apart), the second response was reduced, indicative of receptor desensitization, and was reversed by cyclothiazide in a concentration-dependent manner (1–300 μM). The concentration-response curve for AMPA-induced release of [^{3}H]-noradrenaline was shifted leftwards, but the reversal by cyclothiazide of the desensitized response was partial and failed to reach the maximal response of the first stimulus.

4 Observations made with various schedules of cyclothiazide application indicated that the initial AMPA-evoked response was already partially desensitized (150% potentiation by 100 μM cyclothiazide) and that the desensitization was not likely to be due to a time-dependent diminution and was long-lasting (second application of cyclothiazide was ineffective).

5 Co-application of a number of drugs with actions on second messenger systems, in association with the second AMPA stimulus, revealed significant potentiation of the AMPA-induced release of [^{3}H]-noradrenaline: forskolin (10 μM, +78%), Rp-cAMPS (100 μM, +65%), Ro 31-8220 (10 μM, + 163%) and thapsigargin (100 μM, +161%).

6 The AMPA receptor-mediated response regulating the release of [^{3}H]-noradrenaline from rat hippocampal slices was desensitized and cyclothiazide acted to reverse partially the desensitization in a concentration-dependent manner. Since the time-course of desensitization was longer lasting than that noted in previous electrophysiological studies, multiple events may be involved in the down-regulation of AMPA receptor activity including receptor phosphorylation and depletion of intracellular Ca^{2+} stores.

Keywords: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor; cyclothiazide; desensitization; release

Introduction

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of ionotropic L-glutamate receptor is now recognized to be a multi-domained receptor-ionophore complex, where a range of drugs appear to affect discretely receptor function (Lodge & Palmer, 1993; Zorumski *et al*., 1993; Fletcher & Lodge, 1996). High affinity AMPA receptors, formed from combinations of the subunits GluR1-GluR4, undergo rapid desensitization to AMPA and L-glutamate (Sommer *et al*., 1990; Trussell *et al*., 1993). Cyclothiazide, a thiazide diuretic, potently inhibits such AMPA receptor desensitization in rat cultured hippocampal neurones (Yamada & Tang, 1993; Zorumski *et al*., 1993) by acting to increase the frequency of ion channel opening and the duration of spontaneous miniature excitatory postsynaptic currents (Trussell *et al*., 1993; Yamada & Tang, 1993). Partin *et al*. (1993) have found that cyclothiazide acts only on the AMPA receptor subunits GluR1-GluR4.

AMPA-selective antagonists thus far discovered appear to act at one of two sites on the AMPA receptor complex. 2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX; Sheardown *et al*., 1990) and (−)-(3**SR**,4a**RS**,6**RS**,8a**RS**)-6-[2(1H-tetrazol-5-yl)ethyl]-decahydroisoquinoline-3-carboxylate (LY 293558; Ornstein *et al*., 1995; Schoepp *et al*., 1995) act at the primary acceptor site, whilst 1-(4-aminophenyl)-4-methyl-7,8-methylendioxy-5H-2,3-benzodiazepine (GYKI 52466) seems to antagonize responses to AMPA in a non-competitive fashion at a previously unrecognized site, since the antagonism by GYKI 52466 is not voltage- or use-dependent, although it may affect desensitization kinetics (Donevan & Rogawski 1993; Rammes *et al*., 1996). A body of evidence also supports the existence of a further antagonist site for insect venoms (Fletcher & Lodge, 1996).

Ionotropic glutamate receptors regulating transmitter release can be conveniently studied in a model system where hippocampal slices, loaded with [^{3}H]-noradrenaline (NA), are superfused with glutamate agonists and the stimulus-evoked release of [^{3}H]-NA monitored (Fink *et al*., 1992; Desai *et al*., 1994). Using such an experimental procedure employing consecutive applications (Starke, 1981) of AMPA, we showed that cyclothiazide prevents the desensitization of AMPA receptors regulating the release of [^{3}H]-NA from hippocampal slices, an effect that involves a concentration-dependent potentiation of the AMPA response. Our results also provide evidence for the involvement of phosphorylation and intracellular calcium stores in this long-lasting desensitization of the AMPA receptor.

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Methods

Preparation of hippocampal slices

All animal experimentation was performed according to the code of ethics approved by the National Health and Medical Research Council (Australia). Male Sprague-Dawley rats (200 – 250g) were obtained from the Department of Pharmacology Animal House, Monash University. Rats were killed and brains were rapidly dissected out and cooled on ice. Hippocampi were removed and sliced into prisms (400×400 μm) with a McIlwain tissue chopper. Routinely, the hippocampi from two rats were used in a single experiment. Slices were incubated in 5 ml Krebs-Henseleit (KH) buffer (NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $CaCl_2.2H_2O$ 1.3 mM, D(+)-glucose 11 mM with $MgSO_4.7H_2O$ 1.2 mM) for 10 min at 37°C and gassed with 95% O_2/5% CO_2. Slices and KH buffer were centrifuged (500*g*, 50 s) and the supernatant layer was removed with a Pasteur pipette and discarded.

Incubation with radiolabelled neurotransmitter

The hippocampal prisms were resuspended in 5 ml gassed KH buffer and incubated with 2.5 μl [^{3}H]-NA (1 μCi μl^{-1}) for 30 min at 37°C. Pargyline (1 μM), EDTA (30 μM) and ascorbate (60 μM) were included in the KH buffer during the incubation of the prisms with [^{3}H]-NA (0.05 μM, 30 min, 37°C) and during the release protocol as described previously (Jones *et al*., 1994; 1995), to prevent the metabolism and oxidation of [^{3}H]-NA. The incubation was terminated by gentle centrifugation twice (500*g*, 50 s), and the supernatant layer containing excess ^{3}H neurotransmitter was discarded.

Superfusion and collection period

The prisms were resuspended in 1 ml of KH buffer by gentle agitation with a pasteur pipette and 200 μl portions of this suspension were placed in the chambers of a Brandel Superfusion 600 instrument. The hippocampal prisms were then superfused with buffer at 0.5 ml min^{-1} for a 60 min washout period, with an additional 2 min stimulation with 30 mM K^+-enriched buffer, used to expedite the removal of loosely associated [^{3}H]-NA. Sequential 3 min fractions were collected for 1 h. Slices were stimulated at fractions 4 (S_1) and 13 (S_2) for 2 min with buffer containing AMPA (1 – 1000 μM). Drugs were included at fraction 9 and were superfused until the end of S_2 or for the remainder of the experiment.

Measurement of neurotransmitter release and data analysis

At the end of the experiment, the prisms were removed from the superfusion chambers and the [^{3}H]-NA remaining in the prisms and present in each sample was determined by scintillation spectrometry (Packard Minaxi-Tri-carb 4000 series liquid scintillation counter). Under these experimental conditions the ^{3}H essentially represents unmetabolized [^{3}H]-NA (Jones *et al*., 1995). Release of [^{3}H]-NA in each sample was expressed as the fraction (%) of [^{3}H]-NA in each sample relative to the tissue total at that time. The two fractions preceding S_1 and S_2 were used to determine basal release (R_1 and R_2). AMPA-induced release was taken as including the four fractions following and including the AMPA stimuli. Drug effects were examined by expressing the fractional release data as S_1/R_1, S_2/R_2, S_2/S_1 and R_2/R_1 ratios (Starke, 1981).

Materials

NA (1-[ring-2,5,6-^{3}H], 54 Ci mmol^{-1}) was from NEN Research Products (Wilmington, DE, U.S.A). Tetrodotoxin was obtained from Sapphire Biochemicals, Sydney, Australia. The following drugs were gifts: cyclothiazide salt and LY 293558.H_2O (Eli Lilly, IND, U.S.A.), NBQX (Novo, Malov, Denmark), and Ro 31-8220 (3-[8-(aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione); (Roche, Welwyn, U.K.). GYKI 52466.HC1 was purchased from Research Biochemicals International (Natick, MA, U.S.A.). Other materials were from commercial suppliers.

Statistics

Statistical significance of data was determined by Mann-Whitney U tests for solitary tests or Kruskal-Wallis non-parametric ANOVA, with *post-hoc* Dunn's *t* tests. Where two-way comparisons were performed, a Bonferroni correction for multiple tests was used. A significance level of $P < 0.05$ was used.

Results

Characteristics

AMPA (1 – 1000 μM), when applied during S_1, produced concentration-dependent increases in the release of [^{3}H]-NA (Figure 1a). At concentrations above 30 μM, AMPA-stimulated release of [^{3}H]-NA was significantly reduced during S_2 relative to S_1 (Figure 1b). Basal release (R_2/R_1 ratios) was not significantly affected by any AMPA concentration tested (range of values 0.96 – 1.23).

A series of experiments was performed to establish the characteristics of the AMPA-induced release of [^{3}H]-NA. First, AMPA-stimulated release of [^{3}H]-NA was stereospecific as its inactive enantiomer (**R**)-AMPA (50 μM) when included in S_2 failed to release [^{3}H]-NA, whilst the active enantiomer (**S**)-AMPA (50 μM in S_2) was approximately as effective as 100 μM (**RS**)-AMPA (S_2/S_1 ratios (**RS**)-AMPA 0.285 ± 0.021, (**R**)-AMPA 0.010 ± 0.006 ($P < 0.05$), (**S**)-AMPA 0.227 ± 0.022, $n = 4 – 6$). Secondly, the AMPA-induced release of [^{3}H]-NA was significantly inhibited by superfusing the prisms with Ca^{2+}-free KH buffer or by the inclusion of 1 μM tetrodotoxin (Table 1). Thirdly, the release mechanism was established as AMPA receptor-mediated, since the AMPA (100 μM)-induced release of [^{3}H]-NA was also greatly reduced by each of the competitive AMPA receptor antagonists, LY293558 and NBQX, and by the non-competitive receptor antagonist GYKI 52466, in an apparently concentration-dependent manner (Table 1).

Actions of cyclothiazide

Although AMPA (1 – 1000 μM)-stimulated release was reduced during S_2 relative to S_1 consistent with AMPA receptor desensitization, the S_2 response was still concentration-dependent ($P < 0.05$), but appeared to reach a maximum at lower concentrations of AMPA (Figure 1a). Inclusion of cyclothiazide (100 μM), during the S_2 AMPA depolarization, attenuated the desensitization above 1 μM AMPA, and initially increased the release of [^{3}H]-NA in S_2 above that elicited in S_1. The AMPA concentration-response curve was shifted to the left, although the effect reached a plateau below

the maximal AMPA-induced release (Figure 1a). For ease of interpretation, data were plotted as S/R ratios – although theoretically this value will be influenced by fluctuations in basal release, in practice R_2/R_1 was very constant (range 0.98 – 1.23) and from the same population ($P>0.05$). At 30 μM AMPA, cyclothiazide (100 μM) increased the AMPA-evoked release of [^{3}H]-NA (S_2/S_1 3.9 $\pm$ 1.5 ($n=4$)) to 680% above control (S_2/S_1 0.50 $\pm$ 0.09 ($n=4$); $P<0.05$). The vehicle (buffer containing 0.1% dimethylsulphoxide) was ineffective (S_2/S_1 0.66 $\pm$ 0.08 ($n=4$)) (Figure 1b). Cyclothiazide, at all concentrations tested (1 – 300 μM), elevated AMPA (100 μM)-evoked release of [^{3}H]-NA, an effect that was concentration-dependent (ANOVA, $P<0.05$). Large, significant effects of cyclothiazide were found in the concentration range (30 – 300 μM) when the thiazide was included with AMPA in S_2 ($P<0.05$). However, at higher concentrations of cyclothiazide (100 and 300 μM), there was a small change in basal release of [^{3}H]-NA as reflected by R_2/R_1 ratios (Table 2).

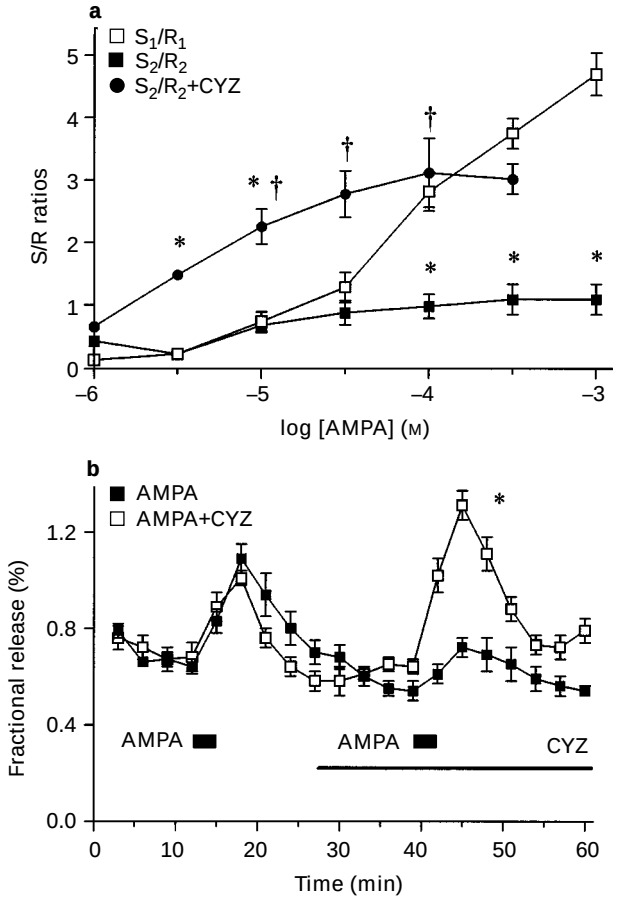

Figure 1 (a) Inhibition by cyclothiazide (CYZ) of the desensitization of the AMPA (1 – 1000 μM)-induced release of [^{3}H]-noradrenaline from rat hippocampal prisms. (a) Presents the 1st AMPA stimulus relative to basal release (S_1/R_1), 2nd AMPA stimulus relative to basal release (S_2/R_2) showing desensitization and 2nd AMPA stimulus relative to basal release (S_2/R_2) in the presence of cyclothiazide (CYZ, 100 μM). Values are the mean of 4 – 13 experiments; vertical lines show s.e.mean. *Significant differences between S_2/R_2 and S_1/R_1 ratios ($P<0.05$), and †significant effect of cyclothiazide on S_2/R_2 ratios ($P<0.05$) (Kruskal-Wallis non-parametric ANOVA, *post hoc* Dunn's *t* test). In all experiments R_2/R_1 ratios were in the range 0.98 – 1.23. (b) AMPA (30 μM)-stimulated release of [^{3}H]-noradrenaline was potentiated by cyclothiazide (100 μM) present during S_2. *A significant potentiation ($P<0.05$, Mann-Whitney U test) by cyclothiazide was observed.

Characteristics of AMPA-cyclothiazide interaction

The schedule of exposure to cyclothiazide was investigated to provide insights into its potentiation of the AMPA-mediated response (Table 3; Figure 2). Although the AMPA (100 μM) response in S_1 was noted above to be appreciably greater than the desensitized response in S_2, the inclusion of cyclothiazide (100 μM) in S_1 resulted in an approximate 2 fold increase in the S/R ratio (121% potentiation; Table 3; Figure 2b) – a potentiation very similar to that found when cyclothiazide was present in S_2 alone (146%; Table 3; Figure 2a). Other factors were examined including time course and the effect of a general depolarization. A single AMPA stimulus, delayed until fraction 13, produced a S/R ratio (3.1 $\pm$ 0.2; Table 3; Figure 2c) that was not significantly different from the standard (100 μM

Table 1 Characteristics of AMPA-induced release of [^{3}H]-NA from hippocampal slices

Treatment	S_2/S_1	R_2/R_1
Control	0.350 $\pm$ 0.031 (100)	0.903 $\pm$ 0.019 (100)
Ca^{2+} free	0.064 $\pm$ 0.038 (18)*	0.752 $\pm$ 0.060 (83)
Tetradotoxin (1 μM)	0.029 $\pm$ 0.017 (8)*	0.564 $\pm$ 0.094 (62)*
LY 293558 (1 μM)	0.189 $\pm$ 0.031 (54)	0.914 $\pm$ 0.043 (101)
LY 293558 (10 μM)	0.052 $\pm$ 0.029 (15)*	1.055 $\pm$ 0.096 (117)
NBQX (1 μM)	0.154 $\pm$ 0.033 (44)	0.879 $\pm$ 0.039 (97)
NBQX (10 μM)	0.075 $\pm$ 0.032 (21)*	0.922 $\pm$ 0.074 (102)
GYKI 52466 (10 μM)	0.386 $\pm$ 0.072 (110)	0.833 $\pm$ 0.030 (92)
GYKI 52466 (30 μM)	0.072 $\pm$ 0.026 (21)*	0.896 $\pm$ 0.050 (99)

Effects of various drugs on the AMPA (100 μM)-stimulated release of [^{3}H]-noradrenaline from hippocampal prisms. Two AMPA stimuli were applied (S_1 and S_2) and treatments were applied during S_2 (from fraction 9 for remainder of experiment) as described in Methods. S_2/S_1 and R_2/R_1 ratios reflect treatment effects on stimulated and basal release, respectively. Values are the mean $\pm$ s.e.mean of 4 – 13 experiments and are given as % control in parentheses. *Significant difference from control ($P<0.05$; Kruskal-Wallis nonparametric ANOVA with a *post-hoc* Dunn's test).

Table 2 The effects of cyclothiazide on AMPA-induced release of [^{3}H]-NA

Cyclothiazide (μM)	S_2/S_1	R_2/R_1
Control	0.350 $\pm$ 0.031 (100)	0.903 $\pm$ 0.019 (100)
1	0.568 $\pm$ 0.060 (162)	0.842 $\pm$ 0.018 (93)
3	0.566 $\pm$ 0.073 (162)	0.796 $\pm$ 0.084 (88)
10	0.727 $\pm$ 0.095 (208)	0.909 $\pm$ 0.028 (101)
30	0.767 $\pm$ 0.101 (219)*	0.978 $\pm$ 0.065 (108)
100	1.272 $\pm$ 0.110 (363)*	1.110 $\pm$ 0.052 (123)*
300	1.294 $\pm$ 0.313 (370)*	1.257 $\pm$ 0.115 (139)*

Effects of cyclothiazide (1 – 300 μM) on the AMPA (100 μM)-stimulated release of [^{3}H]-noradrenaline from hippocampal prisms. Details are given in Table 1. Values are the mean $\pm$ s.e.mean of 4 – 13 experiments and are given as % control in parentheses. Cyclothiazide exerted a concentration-dependent effect on the AMPA (100 μM)-stimulated release of [^{3}H]-noradrenaline (ANOVA, $P<0.05$). *Significant difference from control ($P<0.05$; Kruskal-Wallis nonparametric ANOVA with a *post-hoc* Dunn's test).

AMPA) S_1/R_1 ratio (3.3 ± 0.3) indicating that the desensitization observed in S_2 relative to S_1 was not due to a time-dependent diminution of the AMPA-mediated response over the course of the experiment. The reduction may be due to depolarization during S_1 because a high K^+ stimulus (30 mM) in S_1 resulted in 100 μM AMPA producing a S/R response (1.8 ± 0.3; Table 3; Figure 2d) that, although greater, was nevertheless not significantly different from the control S_2/R_2 response (1.3 ± 0.1).

However, our data do not exclude the possibility of an AMPA- or other ion channel-mediated depolarization inducing a second messenger cascade that in turn affects the AMPA receptor. Interestingly, Desai *et al.* (1994) found that concurrent application of cyclothiazide had no potentiating effect on veratridine- and 4-aminopyridine-induced release. Evidence for a long-lasting desensitization process that might involve second messenger systems was provided by repeating the application of cyclothiazide (after its inclusion in S_1), which was now essentially unable to potentiate the AMPA response in S_2. Finally, the specificity of the AMPA response was studied in a further series of experiments by use of the kainoid, domoate. Similar results were obtained for domoate (30 μM) with cyclothiazide (100 μM) present only during S_1 and when present during S_2 only (Table 4). Since the action of cyclothiazide is restricted to AMPA-preferring receptor subunits (Partin *et al.*, 1993), these are likely to be the site of action of domoate in this experimental paradigm.

AMPA desensitization and second messengers

In view of the preceding evidence for a desensitized AMPA response, which differed from the time-course noted in electrophysiological studies (100–150 ms; Zorumski & Thio, 1992; Rammes *et al.*, 1996), a study was undertaken of the involvement of second messenger systems in the long-lasting desensitization. Additionally, phosphorylation of the AMPA-gated channel has been shown to modulate its activity with the adenosine 3′,5′-monophosphate-dependent protein kinase A (PKA) being especially implicated (Greengard *et al.*, 1991; Wang *et al.*, 1991; Blackstone *et al.*, 1994; Rosenmund *et al.*, 1994; Tan *et al.*, 1994). A number of agents, when superfused before and during the S_2 application of AMPA, potentiated the release of [^{3}H]-NA relative to control experiments (Figure 3, Table 5): forskolin (10 μM, +78%), Rp-cAMPS (100 μM, +65%), Ro 31-8220 (10 μM, +163%) and thapsigargin (100 μM, +161%). Rp-cAMPS (Rp-adenosine 3′,5′-cyclic monophosphothioate) is a specific cyclic AMP inhibitor (Wang *et al.*, 1991), whereas Ro 31-8220 (3-[8-(aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione) is a selective protein kinase C inhibitor (Bit *et al.*, 1993). Phorbol 12-myristyl 13-acetate (100 μM) produced a 23% potentiation of the AMPA response which failed to achieve significance ($P > 0.05$). The following treatments were ineffective: forskolin (1 μM), Rp-cAMPS (10 μM), Ro 31-8220 (1 μM), thapsigargin (10 μM), KN-62 ((**S**)-5-isoquinolinesulphonic acid, 4-[2-[(5-isoquinolinylsulphonyl) methylamino]- 3- oxo -3- (4-phenyl-1-piperazinyl) -propyl]phenyl ester, a selective Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, Tokumitsu *et al.*, 1990; 10 μM), KT-5823 ((8**R**,9**S**,11**S**)-(−)-2,8-dimethyl-9-methoxy-9-methoxycarbonyl - 2, 3, 9, 10 - tetrahydro - 8, 11-epoxy -1H, 8H, 11H-2,7b,11a-triazadibenzo(a,g)cycloocta(c,d,e)trinden-1-one, a selective inhibitor of cyclic guanosine monophosphate-dependent protein kinase, Ito & Karachot, 1990; 10 μM), lavendustin (10 μM), SIN-1 (3-morpholinosydnonimine, a nitric oxide donor; 100 μM), N_G-nitro-L-arginine (100 μM), arachidonic acid (10 μM), and PAF (platelet activating factor, MacLennan *et al.*, 1996; 1 μM). Ro 31-8220 (10 μM) had a significant effect on the spontaneous release of [^{3}H]-NA, increasing the R_2/R_1 ratio 36% above control values (Table 5).

Table 3 Characteristics of release of [^{3}H]-NA from hippocampal slices

Treatment	S_1/R_1	S_2/R_2	R_2/R_1
AMPA 100 μM (S_1 and S_2)	3.3 ± 0.3	1.3 ± 0.1	0.90 ± 0.01
AMPA 100 μM in S_2 alone	*−0.2 ± 0.1*	3.1 ± 0.2*	0.82 ± 0.03
K^+ 30 mM in S_1, AMPA 100 μM in S_2	*14.3 ± 1.4*	1.8 ± 0.3	1.02 ± 0.05*
AMPA 100 μM (S_1 and S_2) CYZ 100 μM in S_2	2.7 ± 0.3	3.2 ± 0.4*	1.06 ± 0.05*
AMPA 100 μM (S_1 and S_2), CYZ 100 μM (S_1 and S_2)	7.3 ± 0.8*	1.3 ± 0.2	1.11 ± 0.04*

Effects of various conditions on the release of [^{3}H]-noradrenaline from hippocampal prisms. One or two stimuli were employed (S_1 and S_2) and treatments were applied as described in Methods (CYZ: cyclothiazide). Values are the mean ± s.e.mean of 6–13 experiments. *Significant difference from S_1/R_1, S_2/R_2 or R_2/R_1 values, respectively, obtained with 100 μM AMPA (S_1 and S_2) ($P < 0.05$; Kruskal-Wallis nonparametric ANOVA with a *post-hoc* Dunn's test). Values in italics not tested under this regime.

Discussion

In the present study, AMPA-induced release of [^{3}H]-NA from hippocampal slices was characterized as an AMPA receptor-mediated event and found to be concentration-dependent, not maximal at 1 mM AMPA, calcium-dependent and tetrodotoxin-sensitive, in agreement with previous observations (Fink *et al.*, 1992; Desai *et al.*, 1994; 1995). By use of an experimental protocol that was quite different to that previously employed to characterize the AMPA-cyclothiazide interaction (Desai *et al.*, 1994; 1995), consecutive AMPA stimuli (S_2/S_1 method; Starke, 1981) revealed a long-lasting desensitization which was cyclothiazide-sensitive. Desai and colleagues (1994, 1995) used a single stimulus S_1 and our results confirm and extend their observations. Thus we were able to demonstrate clearly *in vitro* desensitization to the action of AMPA because cyclothiazide inhibited AMPA receptor desensitization such that initially, the AMPA-stimulated release of [^{3}H]-NA was potentiated. In particular, our findings allow insights into the mode of action of cyclothiazide at the AMPA receptor: first, cyclothiazide shifted the AMPA concentration-response curve to the left, but never fully overcame the desensitization, although an initial potentiation was observed, and secondly, the cyclothiazide effect was concentration-dependent and maximal at 100 μM. These data are in agreement with similar findings in electrophysiological experiments employing thiazide diuretics (Trussel *et al.*, 1993; Yamada & Tang, 1993; Zorumski *et al.*, 1993). The potentiation observed with cyclothiazide at intermediate concentrations of AMPA is consistent with recent evidence (Lodge & Palmer, 1993; Yamada & Tang, 1993), including the demonstration that it also occurs under experimental conditions in which only a single AMPA stimulus was employed (Desai *et al.*, 1994; 1995).

The data obtained with the antagonists are also in general agreement with previous results. While NBQX has not been

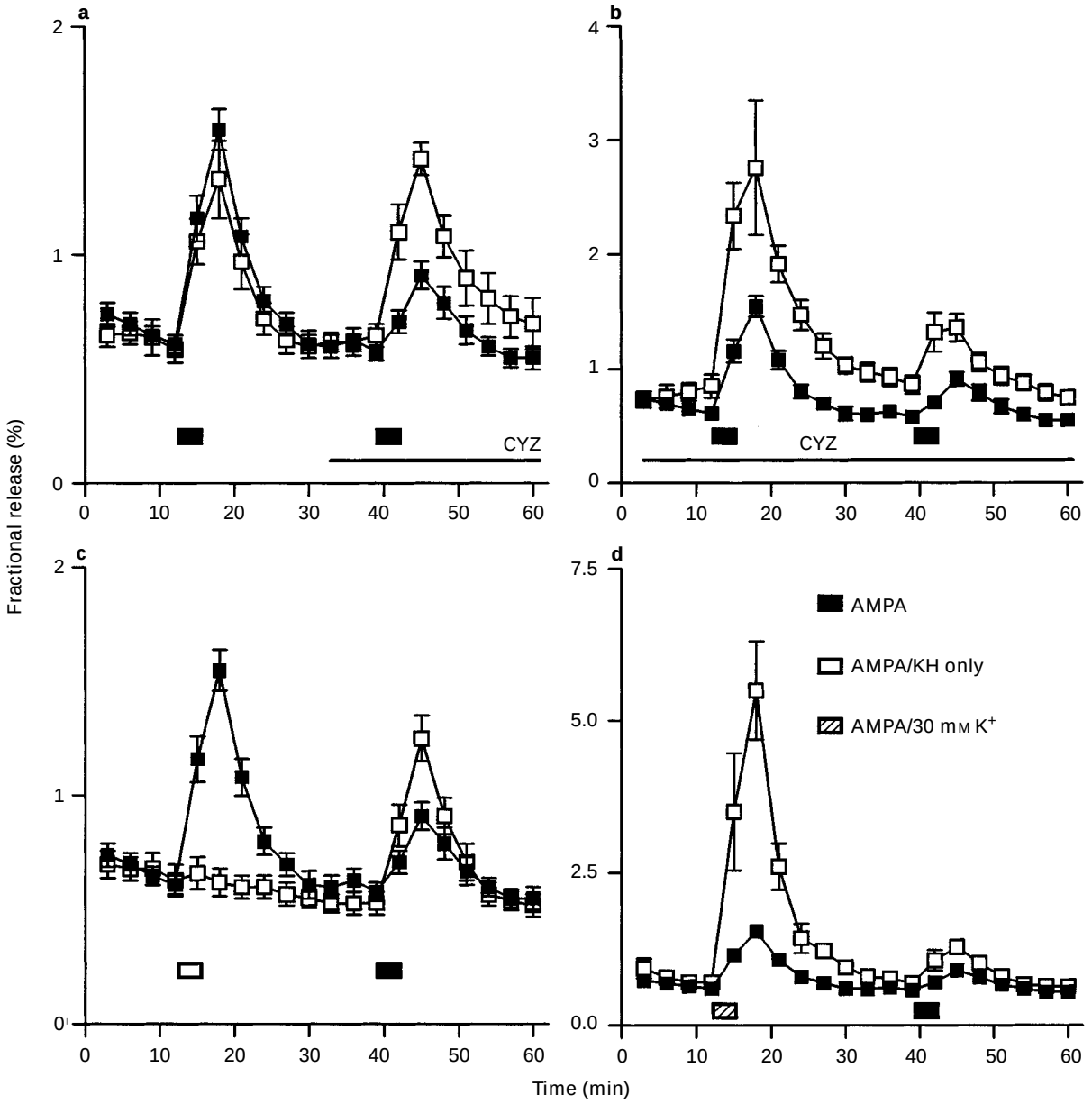

Figure 2 AMPA (100 μM)-induced release of [^{3}H]-noradrenaline (solid squares), showing the effects of various manipulations (open squares) as indicated, (a) cyclothiazide (CYZ; 100 μM) present during S_2, (b) cyclothiazide (100 μM) present during S_1 and S_2, (c) no stimulus in S_1, 100 μM AMPA present in S_2 and (d) high K^+ (30 mM)-stimulus in S_1, 100 μM AMPA present in S_2. Note the different scales on ordinates. Values are the mean of 6–13 experiments; vertical lines show s.e.mean.

tested in release experiments, it has been shown to be potently effective at inhibiting binding of [^{3}H]-AMPA in cortical membranes (Sheardown *et al*., 1990), and also at inhibiting responses to AMPA in electrophysiological experiments (Randle *et al*., 1993; Parsons *et al*., 1994). The inhibition by NBQX of AMPA-induced release of [^{3}H]-NA occurred at slightly higher concentrations than those shown to be effective in binding or electrophysiological experiments. This result seems to reflect the functional nature of the study performed, as similar results were obtained with another competitive AMPA antagonist, LY 293558 (Table 1; Ornstein *et al*., 1995). The non-competitive antagonist, GYKI 52466, also appeared to be more potent in electrophysiological than in release experiments (Table 1; Donevan & Rogawski, 1993; Parsons *et al*., 1994).

One of the most surprising results obtained was the significant depression of responses in S_2 relative to those in S_1 that occurred at higher concentrations of AMPA. This effect does not appear to be due solely to the cyclothiazide-sensitive desensitization, because 100 μM cyclothiazide, a maximally effective concentration, failed to reverse completely the desensitization, and as indicated by Desai *et al*. (1994), the response in S_1 is already desensitized in this way. We also found that the initial S_1 response was desensitized and the potentiation found here (2 fold) was essentially identical to that observed if cyclothiazide was included only in S_2. However, a new finding indicative of a long-lasting desensitization was that the inclusion of cyclothiazide in S_1 led to it being essentially ineffective in S_2. This extended time-course led us to investigate the mechanism of the AMPA-induced desensitization and the possible involvement of intracellular signalling systems.

The cyclothiazide-sensitive desensitization of the AMPA response described herein is much longer lasting than those

Table 4 Characteristics of domoate (10 μM)-induced release of [^{3}H]-NA from hippocampal slices

Treatment	S_1/R_1	S_2/R_2	R_2/R_1
Domoate 10 μM (S_1 and S_2)	1.6±0.3	0.8±0.1	0.90±0.05
Domoate 100 μM (S_1 and S_2), CYZ 100 μM in S_2	2.3±0.2	3.5±0.3*	1.09±0.05*
Domoate 100 μM (S_1 and S_2), CYZ 100 μM (S_1 and S_2)	5.4±1.0*	0.8±0.1	1.04±0.03*

Effects of cyclothiazide (100 μM) on the domoate (10 μM)-stimulated release of [^{3}H]-noradrenaline from hippocampal prisms (CYZ: cyclothiazide). Values are the mean±s.e.mean of 4 experiments. *Significant difference from S_1/R_1, S_2/R_2 or R_2/R_1 values, respectively, obtained with 10 μM domoate (S_1 and S_2) ($P<0.05$; Kruskal-Wallis nonparametric ANOVA with a *post-hoc* Dunn's test).

generally observed in electrophysiological experiments (Zorumski & Thio, 1992; Yamada & Tang, 1993; Rammes *et al*., 1996), which generally have time-courses measured in milliseconds rather than seconds and minutes, and are fully overcome by cyclothiazide. Therefore, the long-term depression of the AMPA-induced release may in fact represent an apparent down-regulation of receptor function and/or possibly some intracellular signalling mechanism. On the basis of our own evidence and previous observations (Desai *et al*., 1994), such events are likely to be receptor-mediated. While our data support the involvement of phosphorylation and intracellular events, we were not always able to discriminate clearly between effects on the receptor, secondary messenger systems or the release process. The function of AMPA receptor channels is regulated by protein phosphorylation, apparently involving the PKA system, as found here for the desensitization of the AMPA response, rather than other kinases (Blackstone *et al*.,

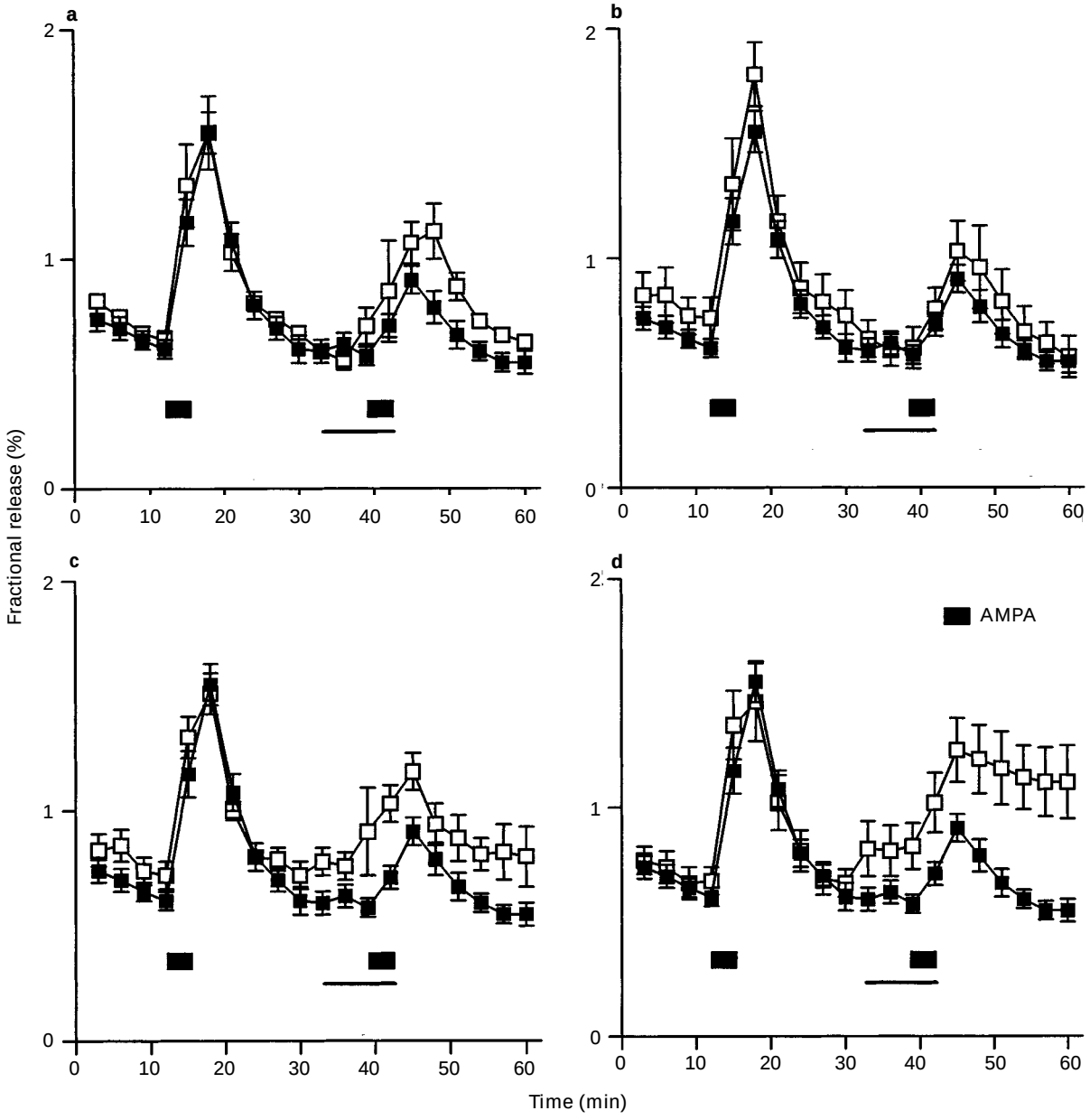

Figure 3 AMPA (100 μM)-induced release of [^{3}H]-noradrenaline (solid squares), showing the effects of modulatory drugs present during S_2 (open squares). (a) Forskolin (10 μM), (b) thapsigargin (100 μM), (c) Rp-cAMPS (100 μM) or (d) Ro 31-8220 (10 μM) was applied at the thin bar. Values are the mean of 4–13 experiments; vertical lines show s.e.mean.

Table 5 The effects of modulatory drugs on AMPA-induced release of [^{3}H]-NA

Drug	*Concentration* (μM)	S_2/S_1	R_2/R_1
Control	–	0.353 ± 0.031 (100)	0.866 ± 0.024 (100)
Forskolin	10	0.628 ± 0.068* (178)	1.021 ± 0.040 (115)
Ro 31-8220	10	0.930 ± 0.253* (263)	1.207 ± 0.110* (136)
Rp-cAMPS	100	0.584 ± 0.059* (165)	0.846 ± 0.047 (95)
Thapsigargin	100	0.920 ± 0.141* (261)	0.960 ± 0.053 (108)

Effects of modulatory drugs on the AMPA (100 μM)-stimulated release of [^{3}H]-noradrenaline from hippocampal prisms. Details are given in Table 1. Values are the mean ± s.e.mean of 4–13 experiments, and are given as % control in parentheses. The following treatments were ineffective: forskolin (1 μM), Ro 31-8220 (1 μM), Rp-cAMPS (10 μM), thapsigargin (10 μM), phorbol 12-myristyl 13-acetate (100 μM), KN-62 (10 μM), KT-5823 (10 μM), lavendustin (10 μM), SIN-1 (100 μM), N_G-nitro-L-arginine (100 μM), arachidonic acid (10 μM) and PAF (1 μM). *Significant difference from control ($P<0.05$; Kruskal-Wallis nonparametric ANOVA with a *post-hoc* Dunn's test).

1994; Tan *et al*., 1994). The regulation of cyclic AMP/PKA system is complex and likely to involve interplay between its catalytic subunit, a regulatory protein and phosphatases, such that a balance is maintained between phosphorylation and dephosphorylation of the AMPA receptor (Wang *et al*., 1991). Theoretically, Rp-cAMPS would be expected to have an opposite effect to forskolin, but both agents attenuated the AMPA desensitization, perhaps reflecting the delicate balance of the multiple mechanisms involved.

Some heteromeric AMPA receptors are Ca^{2+}-permeable (Hollman *et al*., 1991) and cyclothiazide modulates AMPA receptor-mediated rises in intracellular Ca^{2+} (Hoyt *et al*., 1995)–the action of the calcium-mobilizing agent, thapsigargin, thus argues for roles of intracellular calcium pools in the series of events which result in a rather long-term depression of AMPA-mediated function. The selective inhibitor of protein kinase C, Ro 31-8220 (Bit *et al*., 1993), also potentiated the action of AMPA in agreement with the evidence of phosphorylation studies (Blackstone *et al*., 1994; Tan *et al*., 1994) and the existence of consensus phosphorylation sites in AMPA receptor subunits (Hollman & Heinemann, 1994).

The present observations provide new insights into the AMPA-cyclothiazide interaction and desensitization of the AMPA receptor in a functional *in vitro* model. Thus the AMPA-induced release of [^{3}H]-NA is clearly a useful system for examining the AMPA receptor complex, and we were able to demonstrate the presence of several domains of the receptor channel complex in this model. These domains include the cyclothiazide site and the sites for both competitive and non-competitive antagonists. Overall, our findings suggest phosphorylation and intracellular calcium stores are involved in the long-lasting desensitization of the AMPA receptor found in the present experiments. This AMPA-linked desensitization and the involvement of discrete intracellular events bear some similarities to those believed to underlie the phenomenon of long-term synaptic depression (Linden, 1994).

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References

BIT, R.A., DAVID, P.D., ELLIOTT, L.H., HARRIS, W., HILL, C.H., KEECH, E., KUMAR, H., LAWTON, G., MAW, A., NIXON, J.S., VESEY, D.R., WADSWORTH, J. & WILKINSON, S.E. (1993). Inhibitors of protein kinase C. 3. Potent and highly selective bisindolylmaleimides by conformational restriction. *J. Med. Chem.,* **36,** 21–29.

BLACKSTONE, C., MURPHY, T.H., MOSS, S.J., BARABAN, J.M. & HUGANIR, R.L. (1994). Cyclic AMP and synaptic activity-dependent phosphorylation of AMPA-preferring glutamate receptors. *J. Neurosci.,* **14,** 7585–7593.

DESAI, M.A., BURNETT, J.P., ORNSTEIN, P.L. & SCHOEPP, D.D. (1995). Cyclothiazide acts at a site on the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor complex that does not recognize competitive or noncompetitive AMPA receptor antagonists. *J. Pharmacol. Exp. Ther.,* **272,** 38–43.

DESAI, M.A., BURNETT, J.P. & SCHOEPP, D.D. (1994). Cyclothiazide selectively potentiates AMPA- and kainate-induced [^{3}H]norepinephrine release from rat hippocampal slices. *J. Neurochem.,* **63,** 231–237.

DONEVAN, S.D. & ROGAWSKI, M.A. (1993). GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron,* **10,** 51–59.

FINK, K., SCHULTHEIβ, R. & GÖTHERT, M. (1992). Stimulation of noradrenaline release in human cerebral cortex mediated by N-methyl-D-aspartate (NMDA) and non-NMDA receptors. *Br. J. Pharmacol.,* **106,** 67–72.

FLETCHER, E.J. & LODGE, D. (1996). New developments in the molecular pharmacology of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. *Pharmacol. Ther.,* **70,** 65–89.

GREENGARD, P., JEN, J., NAIRN, A.C. & STEVENS, C.F. (1991). Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science,* **250,** 1135–1138.

HOLLMAN, M., HARTLEY, M. & HEINEMMAN, S. (1991). Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science,* **252,** 851–853.

HOLLMANN, M. & HEINEMANN, S. (1994). Cloned glutamate receptors. *Annu. Rev. Neurosci.,* **17,** 31–108.

HOYT, K.R., RAJDEV, S., FATTMAN, C.L. & REYNOLDS, I.J. (1995). Cyclothiazide modulates AMPA receptor-mediated increases in intracellular free Ca^{2+} and Mg^{2+} in cultured neurons from rat brain. *J. Neurochem.,* **64,** 2049–2056.

ITO, M. & KARACHOT, L. (1990). Messengers mediating long-term desensitization in cerebellar Purkinje cells. *NeuroReport,* **1,** 129–132.

JONES, N.M., LOIACONO, R.E. & BEART, P.M. (1995). Role for nitric oxide as an intra- and interneuronal messenger at NMDA release-regulating receptors: evidence from studies of the NMDA-evoked release of [^{3}H]noradrenaline and D-[^{3}H]aspartate from rat hippocampal slices. *J. Neurochem.,* **64,** 2057–2063.

JONES, N.M., LOIACONO, R.E., MØLLER, M. & BEART, P.M. (1994). Diverse roles for nitric oxide in synaptic signalling after activation of NMDA release-regulating receptors. *Neuropharmacology,* **33,** 1351 – 1356.

LINDEN, D.J. (1994). Long-term synaptic depression in the mammalian brain. *Neuron,* **12,** 457 – 472.

LODGE, D. & PALMER, J.P. (1993). Cyclothiazide reverses AMPA receptor antagonism of the 2,3-benzodiazepine, GYKI 53655. *Eur. J. Pharmacol.,* **244,** 193 – 194.

MACLENNAN, K.M., SMITH, P.F. & DARLINGTON, C.L. (1996). Platelet-activating factor in the CNS. *Prog. Neurobiol.,* **50,** 585 – 596.

ORNSTEIN, P.L., ARNOLD, M.B., AUGENSTEIN, N.K., LODGE, D., LEANDER, J.D. & SCHOEPP, D.D. (1993). (3SR,4aRS,6RS,8aRS)-6-[2(1H-tetrazol-5-yl)ethyl]-decahydroisoquinoline-3-carboxylic acid: a structurally novel, systemically active, competitive AMPA receptor antagonist. *J. Med. Chem.,* **36,** 2046 – 2048.

PARSONS, C.G., GRUNER, R. & ROZENTAL, J. (1994). Comparative patch clamp studies on the kinetics of glutamate receptor antagonism by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) and 1-(4-aminophenyl)-4-methyl-7,8-methylendioxyl-5H-2,3-benzodiazepine (GYKI 52466). *Neuropharmacology,* **33,** 589 – 604.

PARTIN, K.M., PATNEAU, D.S., WINTERS, C.A., MAYER, M.L. & BUONANNO, A. (1993). Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A. *Neuron,* **11,** 1069 – 1082.

RAMMES, G., SWANDULLA, D., COLLINGRIDGE, G.L., HARTMANN, S. & PARSONS, C.G. (1996). Interactions of 2,3-benzodiazepines and cyclothiazide at AMPA receptors: patch clamp recordings in cultured neurones and area CA1 in hippocampal slices. *Br. J. Pharmacol.,* **117,** 1209 – 1221.

RANDLE, J.C.R., BITON, C. & LEPAGNOL, J.M. (1993). Allosteric potentiation by diazoxide of AMPA receptor currents and synaptic potentials. *Eur. J. Pharmacol.,* **247,** 257 – 265.

ROSENMUND, C., CARR, D.W., BERGESON, S.E., NILAVER, G., SCOTT, J.D. & WESTBROOK, G.L. (1994). Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. *Nature,* **368,** 852 – 856.

SCHOEPP, D.D., LODGE, D., BLEAKMAN, D., LEANDER, J.D., TIZZANO, J.P., WRIGHT, R.A., PALMER, A.J., SALHOFF, C.R. & ORNSTEIN, P.L. (1995). *In vitro* and *in vivo* antagonism of AMPA receptor activation by (3S,4aR,6R,8aR)-6-[2-(1(2)H-tetrazol-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid. *Neuropharmacology,* **34,** 1159 – 1168.

SHEARDOWN, M.J., NIELSEN, E.Ø., HANSEN, A.J., JACOBSEN, P. & HONORÉ, T. (1990). 2,3,-Dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline: a neuroprotectant for cerebral ischaemia. *Science,* **247,** 571 – 574.

SOMMER, B., KEINÄNEN, K., VERDOORN, T.A., WISDEN, W., BURNASHEV, N., HERB, A., KÖHLER, M., TAKAGI, T., SAKMANN, B. & SEEBURG, P.H. (1990). Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science,* **249,** 1580 – 1585.

STARKE, K. (1981). Presynaptic receptors. *Annu. Rev. Pharmacol. Toxicol.,* **21,** 7 – 30.

TAN, S.E., WENTHOLD, R.J. & SODERLING, T.R. (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *J. Neurosci.,* **14,** 1123 – 1129.

TOKUMITSU, H., CHIJIWA, T., HAGIWARA, M., MIZUTANI, A., TERASAWA, M. & HIDAKA, H. (1990). KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.,* **265,** 4315 – 4320.

TRUSSELL, L.O., ZHANG, S. & RAMAN, I.M. (1993). Desensitization of AMPA receptors upon multiquantal neurotransmitter release. *Neuron,* **10,** 1185 – 1196.

WANG, L.-Y., SALTER, M.W. & MACDONALD, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science,* **253,** 1132 – 1135.

YAMADA, K.A. & TANG, C.-M. (1993). Benzothiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *J. Neurosci.,* **13,** 3904 – 3915.

ZORUMSKI, C.F., YAMADA, K.A., PRICE, M.T. & OLNEY, J.W. (1993). A benzodiazepine recognition site associated with the non-NMDA glutamate receptor. *Neuron,* **10,** 61 – 67.

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