

## ORIGINAL ARTICLE

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## Expression of early hippocampal CA1 LTP does not lead to changes in AMPA-EPSC kinetics or sensitivity to cyclothiazide

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**Abstract** We have analysed whether the expression of long-term potentiation (LTP) in rat hippocampal CA1 neurons involves a change in the kinetics of (*S*)- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory postsynaptic currents (EPSCs) (AMPA-EPSCs) or their susceptibility to the AMPA receptor modulator cyclothiazide. AMPA-EPSCs in the CA1 region were evoked by alternate stimulation of two independent Schaffer collateral-commissural inputs of slices of adult rat hippocampus. In the current-clamp mode a strong tetanus (100 Hz, 1 s) applied to one input (input I) induced stable LTP of AMPA-EPSCs in this input, while the control input (input II) remained unaffected. For neither input were EPSC rise time and decay kinetics significantly changed. The application of cyclothiazide prolonged the rise time and the decay time constants of the AMPA-EPSCs in both control and potentiated inputs to the same extent (Input I—rise time:  $198 \pm 8\%$ , decay:  $148 \pm 12\%$ ; input II—rise time:  $212 \pm 14\%$ , decay:  $144 \pm 19\%$ ;  $n=8$ ). Furthermore, when present during tetanization cyclothiazide did not occlude LTP, suggesting that cyclothiazide and tetanic stimulation enhance AMPA-EPSCs via independent mechanisms. Our findings argue against changes in (de-)activation or desensitization of AMPA receptors as the molecular basis for the expression of LTP.

**Key words** AMPA receptor-mediated EPSCs · Cyclothiazide · Hippocampus · Kinetics · Long-term potentiation (LTP) · Rat

### Introduction

Long-term potentiation (LTP) in the CA1 region of the hippocampus, a phenomenon widely assumed to be a correlate for learning and memory, involves an increase in the amplitude of AMPA [(*S*)- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid] receptor-mediated synaptic responses [11]. This potentiation is probably caused by an increase in the postsynaptic responsiveness of CA1 neurons [14, 23]. This postsynaptic modification probably reflects an increase in receptor number and/or a modulation of receptor properties. Modulatory changes in the properties of AMPA receptors, depending on their nature, could result in a change in the kinetics of synaptic responses. Indeed, it has been reported that LTP induces alterations in the time course of synaptic field potential responses [2], which may reflect changes in the kinetic properties of the AMPA receptors mediating the postsynaptic current response. Possible mechanisms underlying such a change in kinetic properties include the phosphorylation of AMPA receptors [8, 36] as well as a change in the molecular composition of synaptic AMPA receptors [7, 29].

There is evidence that processes which affect AMPA receptor gating kinetics, and thereby alter desensitization and/or deactivation, may be one mechanism for increased AMPA receptor responses underlying the expression of LTP [1]. Collectively, these results suggest that an alteration in AMPA receptor channel kinetics could underly the expression of LTP. Indeed, evidence both for [35] and against [20] such a mechanism has been provided using aniracetam, an agent which enhances AMPA receptor-mediated responses. However, the effects of aniracetam are likely to be mediated through multiple effects on different biological systems (for review see [16]).

The benzothiadiazine cyclothiazide is another positive AMPA receptor modulator, the actions of which have

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meanwhile been extensively investigated [26, 27]. Cyclothiazide greatly potentiates AMPA receptor-induced currents by substantially reducing desensitization and prolongs the decay of AMPA-EPSCs (excitatory postsynaptic currents) by slowing receptor deactivation [26, 27, 28, 37]. These profound effects on AMPA receptor-mediated EPSCs make cyclothiazide an ideal agent to establish whether the expression of LTP involves alterations in AMPA receptor kinetics (deactivation and/or desensitization).

In this study we have analysed whether expression of LTP involves changes in the kinetics of AMPA receptor-mediated EPSCs and we have compared the effects of cyclothiazide on potentiated and non-potentiated synapses in the rat hippocampus. The findings in this study argue against a change in (de-)activation and/or desensitization of AMPA receptors as possible mechanisms for LTP expression.

## Materials and methods

Transverse hippocampal slices (400  $\mu\text{m}$  thick) were obtained from male Wistar rats (140–200 g) which were anaesthetized with ether before decapitation. The brain was removed rapidly and slices were prepared in ice-cold artificial cerebrospinal fluid (aCSF) using a Campden vibroslicer. All slices were placed in a holding chamber for at least 90 min before being transferred to an immersion chamber for recordings. The flow rate of the aCSF through the recording chamber was 1.5 ml/min. The composition of the aCSF was (in mM): NaCl 124, KCl 3,  $\text{NaHCO}_3$  26,  $\text{CaCl}_2$  2,  $\text{MgSO}_4$  1, D-glucose 10,  $\text{NaH}_2\text{PO}_4$  1.25, bubbled with a 95%  $\text{O}_2$ /5%  $\text{CO}_2$  and had a final pH of 7.3.

Patch-clamp recordings were made from pyramidal neurons in the stratum pyramidale of area CA1. The mean input resistance of the cells was  $213 \pm 34 \text{ M}\Omega$ , ( $n=20$ ). Glass electrodes (4.5–5  $\text{M}\Omega$ ) contained (mM):  $\text{CsCH}_3\text{SO}_3$  130, EGTA 0.05, HEPES 5,  $\text{MgCl}_2$  1, NaCl 1, lidocaine *N*-ethyl bromide (QX-314) 5. pH was 7.3, adjusted with KOH. Currents were recorded with a switched voltage-clamp amplifier/SEC 1L (NPI electronic; Tamm/Germany) using the "blind" whole-cell recording technique [10]. Switching frequencies of 20–25 kHz (25% duty cycle) were used. Series resistance was monitored continuously and frequently compensated in bridge mode (for details see [34]). Synaptic currents were generated by test square pulse stimuli (0.033 Hz, 20  $\mu\text{s}$ ) delivered via bipolar tungsten electrodes insulated to the tip (50  $\mu\text{m}$  tip diameter) and positioned in the Schaffer collateral-commissural pathway (Scpp) with two independent inputs converging onto the same cell. In all experiments, the tetanic stimulus pattern to induce LTP was 100 Hz, for 1 s, at control amplitude. The tetanus was delivered in the current-clamp mode. To obtain pure AMPA receptor-mediated EPSCs without blocking *N*-methyl-D-aspartate (NMDA) receptors pharmacologically, neurons were held at a membrane potential of  $-90 \text{ mV}$  in the continuous presence of  $\text{Mg}^{2+}$  (1 mM). In addition slices were perfused with picrotoxin (50  $\mu\text{M}$ ) and 3-amino-propyl (diethoxymethyl)-phosphonic acid (CGP 35348, 200  $\mu\text{M}$ , Ciba Geigy) to block  $\gamma$ -aminobutyric acid ( $\text{GABA}_A$ ) and  $\text{GABA}_B$  receptors, respectively. Voltage-activated sodium and potassium channels were blocked intracellularly by QX-314 and  $\text{Cs}^+$ . All experiments were performed at room temperature. EPSCs were first amplified and filtered (2 kHz) and then digitized (10 kHz) using a laboratory interface (ITC-16 Computer Interface, Instrutech). Digitized current responses were stored to disk on a Macintosh Quadra 700 computer with the acquisition program Pulse v. 7.21 (Heka electronic, Lambrecht, Germany). Single AMPA-EPSCs were well fitted by single exponential func-

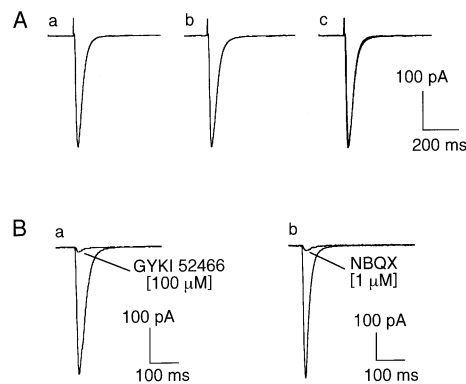
tions to obtain the time constants of decay kinetics. Onset rise time was calculated between 20 and 80% of the peak amplitude. Data are expressed as means  $\pm$  standard error of means (SEM).

The following pharmacological compounds (with sources) were used: all salts and picrotoxin (Sigma), QX-314 and D-AP5 [D(-)-2-amino-5-phosphonopentanoic acid, RBI], CGP 35348. Cyclothiazide (3-bicyclo[2.2.1] hept-5-en-2-yl-6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-solvonamide-1,1-dioxide) was a kind gift from Dr. Leander, Ely Lilly, USA. Stock solutions of cyclothiazide (50 mM) were made in distilled water at pH  $\geq 13$ . After addition to aCSF pH was readjusted to 7.3.

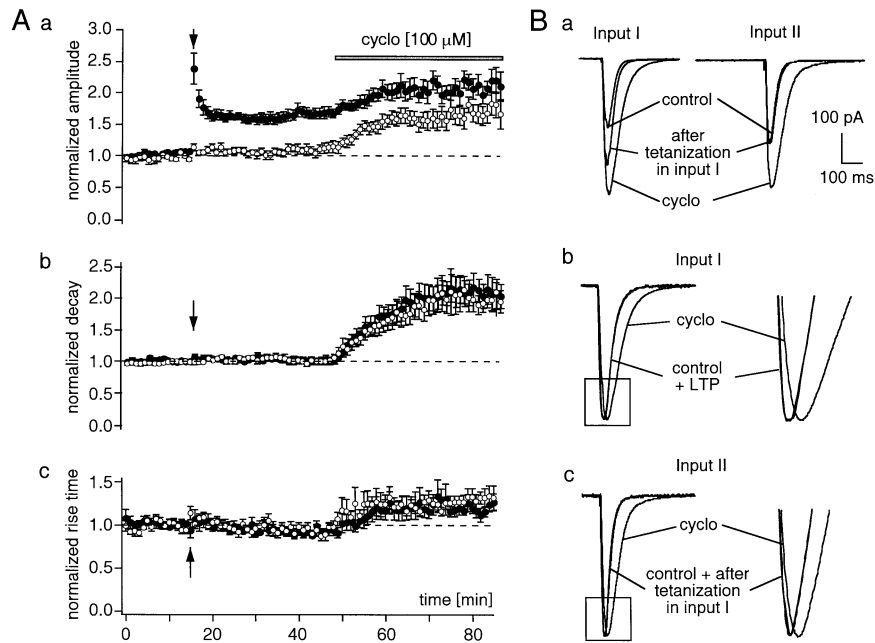
## Results

### LTP expression is not associated with a detectable alteration of AMPA-EPSC kinetics

We first examined whether the expression of LTP is associated with an alteration in the onset and/or offset kinetics of synaptic responses mediated by AMPA receptors. Whole-cell-patch clamp experiments were performed using hippocampal CA1 neurons of young rats, and EPSCs were recorded following extracellular electrical stimulation of two independent Schaffer collateral inputs. To analyse the kinetics of pure AMPA-EPSCs,  $\text{GABA}_A$  and  $\text{GABA}_B$  synaptic components were eliminated with picrotoxin (50  $\mu\text{M}$ ) and CGP 35348 (200  $\mu\text{M}$ ), respectively. Since the activation of NMDA receptors is required for the induction of LTP, NMDA receptors could not be blocked pharmacologically. Instead, neurons were held at a membrane potential of  $-90 \text{ mV}$  in the presence of  $\text{Mg}^{2+}$  (1 mM) to prevent activation of NMDA receptors during low-frequency stimulation. Figure 1 demonstrates that at a holding potential of  $-90 \text{ mV}$ , currents through NMDA receptor channels did not contribute to the EPSCs (five out of five cells). Under these experimental conditions EPSCs were almost completely suppressed by GYKI 1-



**Fig. 1A, B** Excitatory postsynaptic currents (EPSCs) recorded at a membrane potential of  $-90 \text{ mV}$ . **A** EPSCs recorded in the absence (**a**) and presence (**b**) of the *N*-methyl-D-aspartate (NMDA) receptor antagonist D-AP5 (50  $\mu\text{M}$ ). Application of D-AP5 had no effect on either the amplitude or the kinetics of EPSCs, indicating that the recorded EPSCs were pure AMPA-receptor-mediated EPSCs ( $\tau$  decay:  $23.6 \pm 0.54 \text{ ms}$  and  $23.4 \pm 0.76 \text{ ms}$  for control and D-AP5, respectively;  $n=5$ ). **A c** Superposition of the current traces shown in (**a**) and (**b**). **B** EPSCs were blocked by GYKI 52466 (100  $\mu\text{M}$ ) and NBQX (1  $\mu\text{M}$ ) ( $n=7$ )



**Fig. 2A, B** AMPA receptor kinetics and effects of cyclothiazide during expression of early long-term potentiation (LTP). **A** Neurons were held at a membrane potential of  $-90$  mV (in the presence of  $1$  mM  $Mg^{2+}$ ) and synaptic responses were evoked in two independent inputs (LTP input I: filled circles; control input II: open circles). Inhibitory synaptic components were blocked with picrotoxin ( $50$   $\mu$ M) and CGP 35348 ( $200$   $\mu$ M). At the time of the arrowhead a tetanus ( $100$  Hz,  $1$  s) was delivered to input I in current-clamp mode. This induced stable LTP of AMPA-EPSCs in input I, but not in input II (**a**). The decay time constant (**b**) and rise time (**c**) of AMPA-EPSCs remained constant in either input. Thirty minutes after induction of LTP in input I, cyclothiazide ( $100$   $\mu$ M) was applied for  $40$  min as indicated by the bar. The amplitude of both inputs (**a**) was increased to a similar extent (control pathway from  $108 \pm 7\%$  to  $168 \pm 11\%$  and LTP pathway from  $161 \pm 7\%$  to  $213 \pm 12\%$ ;  $n=12$ ). Cyclothiazide increased the decay time constant of the control EPSCs to  $197 \pm 18\%$ , and in the potentiated pathway to  $220 \pm 22\%$  of control (**b**). Cyclothiazide increased the onset rise time of the control EPSCs to  $130 \pm 7\%$  and of the potentiated pathway to  $134 \pm 13\%$  of control (**c**). Each data point was obtained from the average of 2 consecutive EPSCs and normalized to the 15-min period before delivering the tetanus. Mean  $\pm$  SEM; summary of 12 whole-cell experiments. **B** Representative AMPA-EPSCs from a single experiment for input I and input II. Experimental protocol as in Fig. 2A. **B a** Tetanic stimulation of input-I-potentiated responses in this input but not in input II. The subsequent application of cyclothiazide ( $100$   $\mu$ M) increased the magnitude of responses in both pathways to a similar extent. **B b** AMPA-EPSCs were normalized to demonstrate the effects of cyclothiazide on response kinetics. Tetanic stimulation of input I had no effect on the kinetics of AMPA-EPSCs in either input. The subsequent application of cyclothiazide ( $100$   $\mu$ M) slowed onset and offset kinetics in both inputs to similar degrees. Marked (boxes) areas of the current traces were magnified  $3\times$  (right traces) to better resolve current peaks

(4-amino-phenyl)-4-methyl-7,8-methyl-endioxyl-5N-2,3-benzodiazepine 52466 ( $100$   $\mu$ M;  $n=7$ ) and NBQX 2,3-dihydroxy-6-nitro-7-sulfonamyl-benzo(F)quinoxaline ( $1$   $\mu$ M;  $n=7$ ), indicating that they are mediated exclusively by AMPA receptors (Fig. 1Ba, Bb) [27, 28].

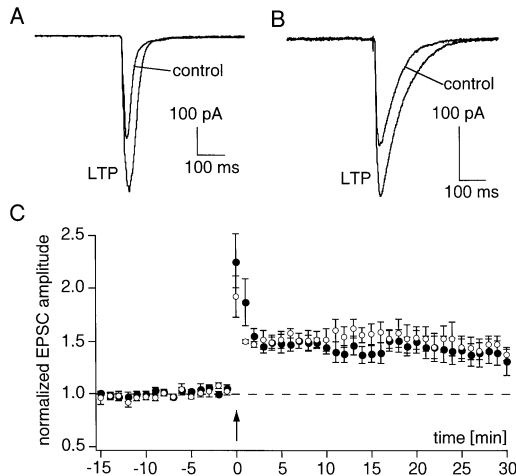
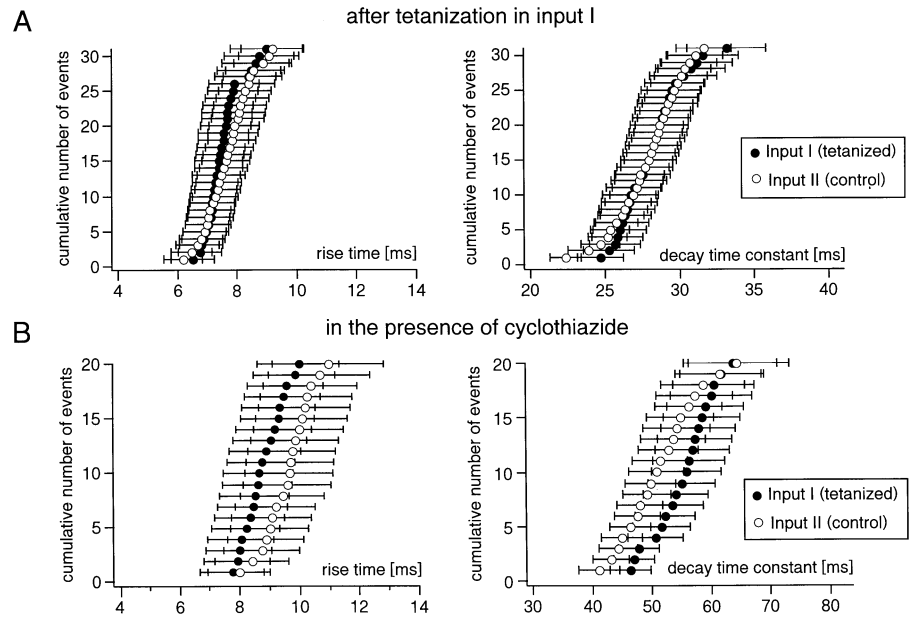
To compare the kinetics of potentiated and control AMPA-EPSCs, we took advantage of the input speci-

ficity of LTP. LTP was generated in one input, whereas the AMPA-EPSCs evoked in a second input were used as control responses. After establishing stable postsynaptic current responses in both inputs for  $15$  min, tetanization ( $100$  Hz for  $1$  s) in one input (input I) resulted in a long-lasting enhancement of synaptic transmission in this input ( $161 \pm 7\%$  of control after  $35$  min; mean  $\pm$  SEM,  $n=10$ ) without potentiation of the second input (input II) (Fig. 2Aa, Ba). The potentiated synaptic responses showed no difference in onset and offset kinetics compared to baseline responses (Figs. 2, 3). Likewise, no alteration of AMPA-EPSC kinetics occurred in input II before and after LTP expression in input I, indicating that no change in recording conditions occurred (Fig. 2). The control AMPA-EPSCs had a rise time ( $20$ – $80\%$ ) of  $8.0 \pm 0.7$  ms and a decay time constant of  $25.8 \pm 1.3$  ms (mean value of  $12$  cells), which is considerably longer than that obtained from dendritic recordings from the same cell type [9]. This difference is probably caused by dendritic filtering of the synaptic current response [25]. Because dendritic filtering could obscure small changes in EPSC kinetics we also used a pharmacological approach to address this issue.

#### Cyclothiazide and LTP enhance AMPA-EPSCs via independent mechanisms

We next tested in the same  $12$  cells if cyclothiazide exerts differential effects on non-potentiated versus potentiated synapses. After  $30$  min of stable LTP in input I, the application of cyclothiazide resulted in similar effects on both pathways. After  $20$  min, cyclothiazide ( $100$   $\mu$ M) increased the amplitudes of the EPSCs in the control pathway from  $108 \pm 7\%$  to  $168 \pm 11\%$ . In the pathway expressing LTP cyclothiazide increased the EPSC amplitudes from  $161 \pm 7\%$  to  $213 \pm 12\%$  (Fig. 2Aa, 2Ba,  $P>0.26$ ,

**Fig. 3A, B** Cumulative histograms of the rise times and decay times of AMPA-EPSCs (summary of 12 experiments, mean $\pm$ SEM) after tetanization in input I (**A**) and in the presence of cyclothiazide (**B**) evoked by stimulation of the tetanized input I (filled circles) and of the control input II (open circles)



**Fig. 4A–C** Enhancement by cyclothiazide of AMPA-EPSCs does not occlude LTP. Representative traces of a single experiment are shown before (*control*) and after LTP induction (*LTP*) without cyclothiazide (**A**) and in the presence of cyclothiazide (**B**). **C** Pooled data from 7 control experiments (black circles) and 7 experiments in which cyclothiazide was present during tetanization (open circles). No significant differences in the extent of synaptic potentiation were observed between the two groups (control:  $146\pm 7\%$ , cyclothiazide:  $146\pm 11\%$ ;  $n=7$ , data taken from the time interval between 25 and 30 min after tetanization). The arrow indicates the time of tetanic stimulation. Each data point represents the average amplitude of 2 consecutive EPSCs normalized to the average EPSC amplitude before tetanization

$n=12$ , Mann–Whitney  $U$ -test). There was no significant difference in the effect of cyclothiazide on AMPA-EPSC rise times (20–80%) and decay time constants in control and potentiated inputs ( $P>0.69$  and  $P>0.55$ , respectively,  $n=12$ ). Cyclothiazide increased the rise time and the decay time constant of control EPSCs to  $130\pm 7\%$  and  $197\pm 18\%$ , and potentiated to  $134\pm 13\%$  and  $220\pm 22\%$  of control, respectively ( $n=12$ ; Figs. 2Bb, 3). Figure 3 shows

cumulative histograms of the rise times and decay time constants of AMPA-EPSCs under control conditions after tetanization in input I and in the presence of cyclothiazide.

#### Cyclothiazide does not occlude the enhancement of AMPA-EPSCs by LTP

In a separate group of experiments we tested whether enhancing AMPA-EPSCs with cyclothiazides occludes LTP. We alternated control experiments with those using cyclothiazide. AMPA-EPSCs were evoked by stimulating the Schaffer collateral pathway in one input. Since the probability of inducing LTP in the whole-cell mode decreases with the duration of whole-cell recording, LTP was induced by tetanization at the same time (30–35 min after obtaining whole-cell access) in all experiments. To ensure a steady-state effect of cyclothiazide, it was applied (together with picrotoxin and CGP 35348) immediately after gaining whole-cell access. In both groups, in two out of seven experiments, tetanizing only induced short-term potentiation (STP) lasting for about 15 min but LTP in the other slices. When all experiments were averaged, the extent of synaptic potentiation was the same in control experiments and in those in which cyclothiazide was present (control:  $146\pm 7\%$ , cyclothiazide:  $146\pm 11\%$ ;  $n=7$ ; Fig. 4).

## Discussion

Our results provide insights into the role of AMPA receptors in the expression of the early phase of LTP. A change in AMPA receptor kinetics may be a molecular basis for the expression of LTP. This is based on the observation that the decay time of synaptic responses is re-

duced after the induction of LTP [2] (but see also [5, 20]). It is well established that protein phosphorylation is essential for the expression of the early phase of LTP (1–3 h; for review see [19]). Furthermore, there is evidence that AMPA receptor phosphorylation is an important mechanism that contributes to the potentiation of synaptic transmission [8, 36]. Two studies have provided evidence that phosphorylation of AMPA receptors enhances synaptic responses in a manner suggesting a change in channel gating kinetics [17, 36]. Our data, however, show that expression of LTP is not accompanied by a change in AMPA-EPSC kinetics. It is still not entirely clear what determines the decay of the AMPA-EPSC in CA1 hippocampal cells. There is evidence for both pre-synaptic and postsynaptic processes determining the decay of the AMPA-EPSC. Several authors have proposed that the decay of the AMPA-EPSC mainly reflects deactivation and hence the off-rate and affinity of L-glutamate [22, 26], but there is also good evidence suggesting that it is primarily determined by asynchronous transmitter release [15]. Our results would therefore argue against both a change in the affinity of the AMPA receptor for L-glutamate and synchronization of transmitter release as the molecular basis of LTP expression.

Other evidence for an involvement of changes in AMPA receptor kinetics in LTP expression came from experiments with the nootropic agent aniracetam. Lynch's group has reported that this drug has different effects on the amplitude and waveform of potentiated versus non-potentiated responses [31]. Other studies, however, detected no interaction between aniracetam-induced changes of the waveform of synaptic events and LTP [6, 20]. Furthermore, the modification of field potential responses described by Ambros-Ingerson et al. [2] could be explained by effects of spike activity rather than by changes in the synaptic response itself [5].

Different effects of AMPA receptor modulators on potentiated versus non-potentiated synapses could, among other things, reflect changes in the composition of AMPA receptors. The differential assembly of subunits from the AMPA subset, designated as GluR1–4 [18], and the alternative splicing of these subunits in the so-called flip and flop splice variants [30] are well-known regulatory mechanisms of AMPA-receptor function. Patch-clamp studies of recombinant receptors support the idea that differential subunit composition and/or splicing may explain the significant differences in AMPA receptor desensitization and deactivation kinetics observed with native AMPA receptors from various regions of the CNS [25, 29]. These differently composed AMPA receptors do not only differ in their kinetics, but are also differently affected by cyclothiazide [21, 25]. Changes in receptor composition could theoretically be involved in the early expression of LTP given that AMPA receptors may be rapidly recycled to and from the synaptic membrane [25a]. However, our results with this drug show that potentiated and non-potentiated synapses are equally affected by cyclothiazide and therefore, together with the lack of change in kinetic properties argue against changes in subunit com-

position of AMPA receptors during expression of early LTP. However, they do not exclude that such changes do occur during the expression of protein-synthesis-dependent late LTP, which is far beyond the usual duration and reliability of whole-cell recordings from the hippocampus.

A previous study with aniracetam [35] (but see [20]) raised the possibility that similar mechanisms may underlie the potentiation of AMPA-EPSCs by nootropic agents such as cyclothiazide and by expression of LTP. In this case one would expect that previous induction of LTP would reduce the effect of subsequent application of cyclothiazide on EPSCs and vice versa. Neither protocol, however, reduced the potentiation of AMPA-EPSCs significantly, suggesting that potentiation of AMPA-EPSCs by cyclothiazide and that by LTP expression do not share common mechanisms.

One would expect that the observed prolongation of AMPA-EPSCs should facilitate the depolarization-induced removal of the  $Mg^{2+}$  block and thereby facilitate LTP induction. IDRA 21, 1-BCP and BDP derivatives have been reported to mediate their facilitatory effects on LTP and learning through inhibition of AMPA receptor desensitization ([3, 4, 32, 33], but see [13]). However, the effects of these drugs on other parameters, such as deactivation kinetics, glutamate release and NMDA receptors, are not well understood and it is still a matter of discussion whether the facilitation of LTP seen with these compounds is caused by modulation of AMPA receptor kinetics. Likewise the observation that cyclothiazide does not induce LTP facilitation may be explained by an antagonizing effect of cyclothiazide on NMDA receptors [15], which may compensate the positive modulation of AMPA receptors during LTP induction. Alternatively, the conditions used in the present experiments may lead to sufficient depolarization in the absence of cyclothiazide for maximum LTP. These results do therefore not preclude the possibility that cyclothiazide can facilitate LTP induced by different protocols.

In summary, our results indicate that changes in the (de-)activation or desensitization behavior of AMPA receptors are not relevant for the expression of LTP. Alternatively, the observed increase in AMPA-receptor-mediated postsynaptic current responses could be caused by a presynaptic alteration in transmitter release probability [12], or a postsynaptic change in AMPA receptor properties such as an increase in the open probability or conductance of the channels [8, 9]. Our observations are also in line with recent findings which support the hypothesis that activation of former non-phosphorylated and non-functional ("silent") receptor channels [5, 24] is an important mechanism for the expression of LTP.

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