EFFECTS OF ACUTE ETHANOL ON THE Ca^{2+} RESPONSE TO AMPA IN CULTURED RAT CORTICAL GABAERGIC NONPYRAMIDAL NEURONS

WOLFGANG FISCHER*, HEIKE FRANKE and PETER ILLES

Rudolf-Boehm-Institute of Pharmacology and Toxicology, University of Leipzig, Germany

(Received 21 June 2002; first review notified 3 September 2002; in revised form 28 March 2003; accepted 20 May 2003)

INTRODUCTION

α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are a subclass of ionotropic glutamate receptors, mediating fast excitatory synaptic transmission in the mammalian brain (for reviews see Michaelis, 1998; Lees, 2000). In distinct cell populations like nonpyramidal neurons of the cerebral cortex and hippocampus, AMPA receptors form ion channels permeable to Ca^{2+}, which seem to play a major role in modulating synaptic plasticity (Geiger et al., 1995; Ozawa et al., 1998; König et al., 2001).

Electrophysiological studies have indicated that ethanol can depress AMPA/kainate-evoked currents in different neuronal preparations using patch-clamp techniques, but these receptors seem to be less sensitive to ethanol than the N-methyl-D-aspartic acid (NMDA) subtype (Lovinger et al., 1989; Weight et al., 1992; Valenzuela et al., 1998). These data conflict somewhat with recent findings showing that AMPA or kainate-activated currents are also strongly inhibited by pharmacologically relevant concentrations of ethanol in cultured hippocampal or medial septum/diagonal band neurons of the rat (Costa et al., 2000; Frye and Fincher, 2000). On the other hand, measurements of AMPA-induced increase in free intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) using fura-2 imaging have shown that acute ethanol application may reduce the amplitude of Ca^{2+} signals in cultured rat cerebellar Purkinje neurons (Gruol et al., 1997). However, the effect of ethanol on AMPA receptor-mediated Ca^{2+} signaling in cortical or hippocampal neurons has hitherto not been investigated.

In the present study we examined the inhibition of (S)-AMPA-induced Ca^{2+} influx by acutely applied ethanol in cultured rat cortical nonpyramidal neurons using single-cell fura-2 microfluorimetry. The AMPA receptor-mediated Ca^{2+} responses in these neurons, identified as GABAergic internune-like cells by immunocytochemistry, have been characterized in detail elsewhere (Fischer et al., 2002).

MATERIALS AND METHODS

Cortical cell cultures

Neuronal cultures were prepared from the cerebral cortex of 16-day-old rat embryos (strain WIST/Lei) by trypsinization/ trituration and cultured in Dulbecco’s modified Eagle medium/ Nutrient mixture F12 (Life Technologies Karlsruhe, Germany; supplemented with 20% fetal heat-inactivated bovine serum, 2.5 mM L-glutamine, 36 mM D(+)-glucose, 15 mM HEPES, 50 μg/ml gentamycin; pH 7.4) on glass coverslips precoated with poly-L-lysine (see Fischer et al., 2002). The cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_{2} and used for experiments after 10–15 days in vitro.

Fura-2 microfluorimetry

Following loading with fura-2/AM (5 μM; see Drugs) in culture medium (37°C, 30 min), the cells were washed in physiological saline (composition in mM: NaCl 133, KCl 4.8, KH_{2}PO_{4} 1.2, MgCl_{2} 1, CaCl_{2} 1.3, HEPES 10, D(+)-glucose 10; pH 7.4; room temperature, 30 min) to remove extracellular traces of the dye. The coverslips were then mounted cell-side up in the bottom of a perfusion chamber (250 μl), placed on the stage of an inverted microscope with epifluorescence optics (Diaphot 200; Nikon, Kanagawa, Japan). Throughout the experiments, the cells were continuously superfused at 0.8 ml/min by means of a roller pump with drug-free or drug-containing solution. In a first series of experiments, the glutamate receptor agonists (AMPA, NMDA or kainate) were applied three times (S_{1}, S_{2}, S_{3}) for 60 s every 10 min by using the roller pump. Ethanol was superfused 10 min before and during S_{2}. In an additional series of experiments, AMPA was applied directly to single cells by pressure for 10 s every 10 min using a DAD12 fast application system (Adams and List, Westbury, NY, USA). Ethanol, tetrodotoxin/bicuculline or nifedipine were superfused 10 min before and during the next application.

*Author to whom correspondence should be addressed at: Rudolf-Boehm-Institute of Pharmacology and Toxicology, University of Leipzig, Hartelstrasse 16–18, D-04107 Leipzig, Germany. Tel.: +49 341 9724603; Fax: +49 341 9724609; E-mail: fisw@medizin.uni-leipzig.de

© Medical Council on Alcohol 2003
of AMPA (in combination with the drug tested) by pressure application. The tissue bath was continuously superfused with drug-free or drug-containing solution (see above), and the solution was removed from the bath with a vacuum pump.

Fura-2 fluorescence (over the cell somata of medium-sized multipolar nonpyramidal neurons), excited alternatively at 340 and 380 nm, was measured at 510/520 nm by a microscope photometer attached to a photomultiplier detection system (Ratiomaster System; PTI, Lawrenceville, NJ, USA). The agonist-induced rise of [Ca\textsuperscript{2+}], was defined as the peak increase in the Δ fluorescence ratio (i.e. the fluorescence ratio 340/380 nm in response to the agonist minus the basal fluorescence ratio) (details of fura-2 microfluorimetry can be found in Fischer et al., 2002). Data acquisition and analysis were performed computer-controlled by using commercially available software (FeliX, Vers.1.1: PTI). Calibration of [Ca\textsuperscript{2+}], was performed according to Grynkiewicz et al. (1985): Ca\textsuperscript{2+}-saturated fura-2 signals (R_{\text{max}}) were determined in the presence of 10 μM ionomycin (Mg\textsuperscript{2+}-free buffer), and Ca\textsuperscript{2+}-free signals (R_{\text{min}}) with 25 mM EGTA (Ca\textsuperscript{2+}-free buffer).

**GABA immunochemistry**

Sister cell cultures were briefly washed with phosphate-buffered saline (0.1 M, pH 7.4), fixed in 4% paraformaldehyde/0.25% glutaraldehyde (4°C, 20 min), treated with 1% H\textsubscript{2}O\textsubscript{2} to inactivate endogenous peroxidase activity and preincubated with a ‘blocking solution’ (10% normal horse serum and 0.1% Triton X-100 in Tris buffered saline) before incubation (4°C, overnight) with the primary antibody (mouse monoclonal anti-GABA antibody, clone 5A9, 1:1000; ICN Biomedicals, Aurora, OH, USA). The preparations were then washed in Tris buffered saline and incubated with the secondary antibody, biotinylated horse anti-mouse immunoglobulin G (1:65; 20°C, 60 min). Following treatment with avidin-biotin complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), the cells were visualized by 3,3′-diaminobenzidine (DAB-Ni/Co; Sigma-Aldrich, Taufkirchen, Germany). The stained cultures were dehydrated in a series of graded ethanol and covered with entellan. Control experiments were carried out without the primary antibody. In some cultures representative fields with living neurons were photographed using an inverted phase-contrast microscope (CK2-TR; Olympus, Hamburg, Germany) before starting GABA-immunocytochemistry. GABA-stained preparations were investigated and photographed using a light microscope (Axioskop 50; Carl-Zeiss Jena, Germany).

**Drugs**

The following drugs were used: (S)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainic acid, NMDA, (+)-bicuculline, EGTA, fura-2 acetoxymethyl ester (Fura-2/AM), ionomycin, nifedipine, tetrodotoxin citrate (TTX) (all from Sigma-Aldrich, Taufkirchen); ethanol absolute (Mallinckrodt Baker; Deventer, The Netherlands); 1-(4-aminophenyl)-4-methyl-1,7,8-dihydroxy-4,5-dihydro-3-methyl-carbamoyl-2,3-benzodiazepine (GYKI 5365; gift of Dr L. Harsing, Institute for Drug Research, Budapest). All other chemicals for the superfusion solutions were obtained from Sigma-Aldrich.

**Statistics**

The data were calculated as means ± SEM of n determinations (individual cells). In all cells included in statistical analysis, the control response to the tested agonist (AMPA, NMDA or kainate) fully recovered following the 10-min-washout period with drug-free solution. The difference between the control response and the response after ethanol superfusion (absolute values) for each cell was compared with the paired Student’s t-test after passing the normality test. Comparison of the inhibitory effects by 100 mM ethanol on AMPA- versus NMDA-induced response (normalized values) was performed by using two-way analysis of variance (ANOVA) with doses as within-subject factor and drug treatment as the between-subject factor. Pairwise multiple comparisons of control response with different drug-modulated response (absolute values) were performed by one-way ANOVA followed by post-hoc Student–Newman–Keuls test. The statistics software used were SigmaStat (Ver. 2.03; SPSS, Erkrath, Germany). A probability level of <0.05 was considered to be statistically significant.

**RESULTS**

The immunocytochemical studies revealed that in our primary cultures of rat cortical cells the vast majority of neurons are GABA-immunopositive with nonpyramidal interneuron-like shape (Fig. 1a,b). For the microfluorimetric investigations, single multipolar (medium-sized) neurons identified morphologically according to the shape of the somata and the divergently branching dendritic trees were selected.

These neurons have average resting [Ca\textsuperscript{2+}], levels of 86 ± 7 nM (n = 10 cells) in the presence of 1.3 mM extracellular Ca\textsuperscript{2+}. The superfusion of ethanol alone had no significant effect on resting [Ca\textsuperscript{2+}]. At 20 mM ethanol did not markedly influence the AMPA (10, 30 and 100 μM)-induced rise in [Ca\textsuperscript{2+}]. Higher concentrations of ethanol tended to decrease (50 mM: statistically significant inhibition only at 10 μM AMPA by 18 ± 3%; n = 7) or even clearly decreased (100 mM) the AMPA induced increase in [Ca\textsuperscript{2+}] by 22 ± 2 (10 μM; n = 8), 17 ± 4 (30 μM; n = 8) and 18 ± 7% (100 μM; n = 6; P < 0.05 each), respectively, without a pronounced dependence on the AMPA concentration tested (see Fig. 2c; summary plot). The original Ca\textsuperscript{2+} responses to AMPA fully recovered upon the washout of ethanol (Fig. 2a). The NMDA (10, 30, 100 μM; plus 10 μM glycine)-induced increase in [Ca\textsuperscript{2+}], was reduced at 100 mM ethanol by 31 ± 6, 24 ± 6 (n = 6; P < 0.05 each) and 22 ± 5% (n = 10; P < 0.05), respectively (Fig. 2b,c). There is no statistically significant difference between the two agonists, the drug × concentration interaction and the various concentrations (two-way ANOVA).

As AMPA receptors can be gated by kainate in a non-desensitizing manner, some studies with this agonist (100 μM) were also performed. The results revealed an inhibition by ethanol (100 mM) of 25 ± 4% (n = 6; P < 0.05). The kainate-induced increase in [Ca\textsuperscript{2+}], was markedly, but not fully suppressed by the selective AMPA receptor antagonist GYKI 53655 (30 μM) (89 ± 5% inhibition; n = 8; P < 0.01).

In the previous microfluorimetric investigations, the cells were superfused with the glutamate receptor agonists (AMPA, NMDA, kainate) for 60 s, every 10 min at a slow rate. In an additional series of experiments, AMPA was applied directly to single cells by fast pressure-application for 10 s, every 10 min. AMPA (30 μM) induced a rapidly rising and declining Ca\textsuperscript{2+} signal, which was reproducible upon repetitive stimulations. First tests with ethanol at 100 mM (superfused 10 min before...
and during the following AMPA (plus ethanol 100 mM)-pressure application) reduced the peak amplitude by 19 ± 3% (n = 9; P < 0.05), well comparable to the value measured previously with the slow superfusion system.

To address the question whether GABA itself induces a possible increase in [Ca\(^{2+}\)]\(_i\) in developing neurons of our cell cultures, the effect of pressure applied GABA (1, 10 and 100 µM) on single cells was investigated. The lowest GABA concentration failed to produce any significant change in fluorescence intensity, but 10 and 100 µM of this agonist induced a rise in Ca\(^{2+}\) response depending on the age of the cultures. In younger cultures (10–12 days in vitro) 17 from 24 (71%) of the neurons tested showed a marked Ca\(^{2+}\) signal. The means of \(\Delta\) fluorescence ratios evoked by GABA (100 µM) and AMPA (30 µM) were 0.78 ± 0.12 and 2.14 ± 0.25 (n = 17 each), respectively. In older cultures (13–15 days in vitro), only in 5 from 21 (24%) neurons a transient Ca\(^{2+}\) signal could be observed. The GABA (100 µM)-induced Ca\(^{2+}\) signals were

Fig. 1. Primary culture of rat cerebral cortical neurons prepared from 16-day-old embryos (14 days in vitro) in a study of the effects of acute ethanol on the Ca\(^{2+}\) response to AMPA in cultured rat cortical GABAergic nonpyramidal neurons. (a) Phase-contrast photomicrograph of living cell culture. Representative field with a characteristic network of well-differentiated neurons. (b) GABA immunostaining of the same field. The vast majority of identified neurons display GABA-positive immunoreactivity. The cell bodies showed a multipolar morphology with an irregular branching pattern of their dendrites. (Bar: 200 µm).

Fig. 2. Influence of ethanol on intracellular Ca\(^{2+}\) responses elicited by superfusion with (S)-AMPA or NMDA in cultured rat cortical nonpyramidal neurons, based on fluorescence ratio measurement. (a) Effect of ethanol (ETOH; 100 mM) on the AMPA (30 µM)-induced Ca\(^{2+}\) signals. (b) Effect of ethanol (ETOH; 100 mM) on the NMDA (30 µM; plus 10 µM glycine, Mg\(^{2+}\)-free solution)-induced Ca\(^{2+}\) signals. Representative tracings from two experiments with individual cells. The glutamate receptor agonists were superfused three times (S1, S2, S3) for 60 s every 10 min. Ethanol was superfused 10 min before and during S2. (c) Summary plot of effects of ethanol on the AMPA- or NMDA-induced Ca\(^{2+}\) signals at the concentrations indicated. All data are expressed as means ± SEM and are calculated as a percentage of the control response at S1. (Numbers of cells are indicated at the base of each column.) The difference between the AMPA- or NMDA-induced signals S1 vs S2 (absolute values) was assessed for statistical significance (paired Student’s t-test). *P < 0.05. No statistically significant differences were evaluated between the mean values of the effects of ethanol (100 mM) on AMPA- and NMDA-induced Ca\(^{2+}\) signals at the concentrations indicated. (fig 2a, b, c).
inhibited by nifedipine (10 µM; 70 ± 3% inhibition, n = 8; P < 0.001) and bicuculline (10 µM; >90% inhibition, only three neurons tested).

However, the superfusion of the GABA_A receptor antagonist bicuculline (10 µM) together with the Na+ channel blocker tetrodotoxin (0.5 µM), to rule out possible effects of spontaneously released GABA and synaptic spike activity in the cell culture, did not significantly modulate the peak amplitude of the AMPA-induced Ca^{2+} signal as well as the inhibitory effects of ethanol on AMPA response (Fig. 3a,c). In these experiments, ethanol inhibited the AMPA response by 20 ± 4% (n = 7; P < 0.05) and the AMPA response following tetrodotoxin/bicuculline superfusion by 23 ± 4% (n = 7; P < 0.05). Noteworthy, in some separate tests, the superfusion of tetrodotoxin/bicuculline (10 µM) alone practically did not alter the AMPA-induced response (105 ± 3%, n = 16; P > 0.05).

Superfusion with the Ca^{2+} channel blocker nifedipine (10 µM) caused a tendency of the AMPA response to decrease by 15 ± 3% (n = 6; P > 0.05). Superfusion with ethanol (100 mM) alone caused an inhibition of 21 ± 4% (n = 6; P < 0.05) and the coapplication of both drugs revealed significant additive inhibitory effects (39 ± 4%, n = 6; P < 0.001) (Fig. 3b,c).

**DISCUSSION**

As we have reported previously, the majority of neurons in our primary cultures of rat cortical cells are GABA- immunopositive, exhibit Ca^{2+}-binding proteins and express Ca^{2+}-permeable AMPA receptors (Fischer et al., 2002). It was further shown by fura-2 microfluorimetry that the stimulation with the selective agonist (S)-AMPA induced a concentration-dependent increase in [Ca^{2+}], which seems to be due to Ca^{2+} entry through the AMPA receptor channels themselves. This assumption is suggested by the findings that the Ca^{2+} response to AMPA was abolished after removal of extracellular Ca^{2+}, and by AMPA receptor selective antagonists, but was not significantly influenced by depletion of intracellular Ca^{2+} stores with cyclapicazonic acid or the application of selective NMDA receptor antagonists, tetrodotoxin and various Ca^{2+} channel blockers (nifedipine, ω-conotoxin MVIIIC or flunarizine) (Fischer et al., 2002). Concerning the NMDA

---

**Fig. 3.** Influence of ethanol on intracellular Ca^{2+} responses elicited by pressure application of (S)-AMPA and modulation of the responses by tetrodotoxin/bicuculline or nifedipine in cultured rat cortical nonpyramidal neurons. (a) Effect of ethanol (ETOH; 100 mM) on the AMPA (30 µM)-induced Ca^{2+} signals and failure of tetrodotoxin (TTX; 0.5 µM)/bicuculline (BIC; 10 µM) to influence the AMPA-induced Ca^{2+} signals as well as the inhibitory effect of ethanol on the AMPA response. (b) Effect of ethanol (ETOH; 100 mM) on the AMPA (30 µM)-induced Ca^{2+} signals and influence of nifedipine (NIF; 10 µM) on the AMPA-induced Ca^{2+} signals as well as on the inhibitory effect of ETOH on the AMPA response. Representative tracings from two experiments with individual cells. In contrast to Fig. 2, AMPA was pressure applied eight times (S1–S8) for 10 s every 10 min. In (a), tetrodotoxin/bicuculline were superfused together 10 min before and during S1 and continuously up to the end of S8 (see the cross-bar on the top) and pressure applied together with AMPA (S1, S2, S3, S4) or AMPA/ETOH (S5; see the small application-bars under the signals); ethanol was superfused 10 min before and during S2 and S6, and pressure applied together with AMPA (S1) or AMPA/ETOH (S5); ethanol was superfused 10 min before and during S1 and S8; (S1, S2, S3, S4) or AMPA/ETOH (S5). In (b), ethanol was superfused 10 min before and during S1 and S6, and pressure applied together with AMPA (S1) or AMPA/ETOH (S5), respectively. In (c), nifedipine was superfused 10 min before and during S1 and S6, and pressure applied together with AMPA (S1) or AMPA/ETOH (S5), respectively. (c) Summary plot. Left-hand panel: Effects of ethanol (ETOH 100 mM) alone and in combination with tetrodotoxin/bicuculline (TTX 0.5 µM/BIC 10 µM) on the AMPA-induced Ca^{2+} signals. Right-hand panel: Effects of ethanol (ETOH 100 mM) alone and in combination with nifedipine (NIF; 10 µM) on the AMPA-induced Ca^{2+} signals. (Numbers of cells are indicated at the base of each column). All data are expressed as means ± SEM and are calculated as a percentage of the corresponding control response before drug superfusion. The differences (absolute values) between S1 vs. S2, S3 vs. S4 and S5 vs. S6 (left) and between S2 vs. S3, S4 vs. S5 (right) were assessed for statistical significance (pairwise multiple comparison by one-way ANOVA followed by Student-Newman-Keuls test). *P < 0.05. The Δ fluorescence ratios evoked by (S)-AMPA (30 µM) at S1 (before 100 mM ethanol) were 2.41 ± 0.49 (n = 7; left) and 2.35 ± 0.43 (n = 6; right), respectively.
(plus glycine)-induced increase in $[\text{Ca}^{2+}]_i$, in both rat cortical and mesencephalic neurons, earlier experiments have also demonstrated that voltage-dependent $\text{Na}^+$ or $\text{Ca}^{2+}$ channels are not involved in the agonist effects (Scheibler et al., 1999).

Previous studies of the inhibitory effect by ethanol on non-NMDA receptor-mediated currents in various neuronal preparations yielded controversial data. For example, in brain slices from rat hippocampus or nucleus accumbens, intoxicating concentrations of ethanol (50–100 mM) exhibit little or no effects on excitatory synaptic transmission mediated by AMPA receptors (Lovingier et al., 1990; Nie et al., 1994; Weiner et al., 1999). Weiner et al. (1999) reported that in rat CA3 pyramidal neurons ethanol depressed pharmacologically isolated kainate and NMDA receptor-mediated excitatory postsynaptic currents (EPSCs), whereas EPSCs mediated by AMPA receptors were not significantly affected even at the highest ethanol concentration tested (80 mM). In cultured rat neuronal cell populations, however, ethanol can significantly depress AMPA/kainate activated currents (Valenzuela et al., 1998; Costa et al., 2000; Frye and Fincher, 2000). Thus, Frye and Fincher (2000; see their fig. 2) observed at 100 mM ethanol about 20–30% inhibition of AMPA (100 nM)- or kainate (100 nM) activated currents in acutely isolated medial septum/diagonal band neurons of the rat, well comparable with our values concerning AMPA-induced $\text{Ca}^{2+}$ signaling. Inhibition by ethanol (25–200 mM) of AMPA and kainate receptor-mediated depolarizations of the hippocampal CA1 area in brain slices was also reported (Martin et al., 1995).

As suggested by various patch-clamp studies, the potency of ethanol to inhibit the NMDA receptor-function appears to be higher than its potency to inhibit AMPA/kainate receptors (Lovingier, 1993; Valenzuela et al., 1998). However, the potency with which ethanol inhibits NMDA receptors also greatly varies between native neurons. Various factors, such as differences in experimental conditions (e.g. buffered medium, temperature, intact or dialysed intracellular milieu) and different neuronal cell preparations (special brain regions, brain slices, cultured neurons with or without glial bed, developing and mature neurons) may contribute to the differences in the inhibitory potency of ethanol for different glutamate receptor-mediated responses (for discussion see Lovingier et al., 1990).

On the other hand, there is very limited information about the inhibitory action by ethanol on AMPA receptor-mediated $\text{Ca}^{2+}$ signaling. In one study, non-NMDA (i.e. quisqualate, kainate)-mediated increases in cytosolic $\text{Ca}^{2+}$ were not affected by a high concentration (100 mM) ethanol in acutely dissociated brain cells from newborn rats (Dildy-Mayfield et al., 1991). In contrast, some non-NMDA and NMDA responses were inhibited by ethanol to a similar extent in oocytes expressing rat hippocampal mRNA (Dildy-Mayfield and Harris, 1992). In another report, ethanol was shown to reduce the amplitude of $\text{Ca}^{2+}$ signals evoked by AMPA in cultured rat cerebellar Purkinje cells (Gruol et al., 1997). A pharmacological characterization of the AMPA receptors involved in the mediation of $\text{Ca}^{2+}$ signals was not performed in this study. However, Gruol and Curry (1995) reported also an enhancement of quisqualate-induced $\text{Ca}^{2+}$ signals following acute ethanol application in cerebellar Purkinje and granule cells.

In the present investigation, we provide evidence that the AMPA receptor-induced increase in $[\text{Ca}^{2+}]_i$ is sensitive to ethanol in cultured rat cortical GABAergic interneuron-like cells. However, the findings indicate that only high concentrations of ethanol inhibit $\text{Ca}^{2+}$ signalling. Our observation that AMPA- and NMDA-induced $\text{Ca}^{2+}$ responses were reduced by ethanol (100 mM) to an almost similar extent, suggested a lack of selectivity of the depressant effects of ethanol on $\text{Ca}^{2+}$ signaling in these cells. However, it is possible that NMDA receptors may be more sensitive than AMPA receptors to concentrations of ethanol lower than 100 mM, which were not tested with NMDA in the present study. Previous findings, however, argued against such an idea. Thus, Bhave et al. (1996) found in rat cortical neuronal cell cultures a threshold concentration of ethanol at about 30 mM for inhibiting the NMDA (100 nM)-induced increase in $[\text{Ca}^{2+}]_i$; the inhibition by ethanol was at 50 mM about 15% and at 100 mM about 35%. Wirkner et al. (1999) measured higher values (~20 and ~40% inhibition at 30 and 100 mM ethanol, respectively) for the inhibition of NMDA (20 µM)-induced $\text{Ca}^{2+}$ signals. Further studies in our laboratory under similar methodical condition as used in the first series of experiments in the present work found for the NMDA (30 µM)-induced $\text{Ca}^{2+}$ response no significant inhibition by 30 mM ethanol, ~20% at 50 mM and ~30% at 100 mM ethanol (A. Kronfeld and C. Allgaier, unpublished results). All these data, when supplemented with our present results indicate a comparable sensitivity of NMDA and AMPA receptor-induced $\text{Ca}^{2+}$ signaling to both high and low concentrations of ethanol.

In agreement with the recently reported effects of ethanol on AMPA-induced currents (Costa et al., 2000; Frye and Fincher, 2000), it can be suggested that both AMPA and NMDA glutamate receptor subtypes are targets of ethanol in diverse cell populations. Moreover, our data are also consistent with the findings of previous whole-cell voltage-clamp experiments showing that ethanol inhibits NMDA and AMPA receptor function by a similar and noncompetitive mechanism (Wirkner et al., 2000). It is of note that the lipophilic trichloroethanol, the main metabolite of chloral hydrate, which more potently (in the low millimolar range) inhibits $\text{Ca}^{2+}$ responses to both AMPA and NMDA than ethanol, also causes similar inhibition of $\text{Ca}^{2+}$ signals to both types of excitatory amino acid agonists (Scheibler et al., 1999; Fischer et al., 2000).

In the first series of experiments, the studies were performed in the absence of tetrodotoxin and the GABA<sub>A</sub> receptor antagonist bicuculline. Since GABA can function as an excitatory neurotransmitter during early neuronal development, inducing membrane depolarization via GABA<sub>A</sub> receptor-activation, and an increase in $[\text{Ca}^{2+}]_i$, as well as firing of action potentials (Owens et al., 1996; Rego et al., 2001; Ben-Ari, 2002), it seemed of interest to carry out new experiments in the presence of tetrodotoxin and bicuculline. However, these drugs did not alter the effect of ethanol in the 10–15-day-old cortical cultures used, although in separate studies, GABA (10–100 µM) induced a transient increase of $[\text{Ca}^{2+}]_i$ in most neurons of younger cell cultures (10–12 days in vitro). Therefore, it is concluded, that neither a possible GABA release in our cell cultures enriched with GABA-immunopositive neurons, nor the additional block of spike activity seems to be of major significance for the $\text{Ca}^{2+}$ signals elicited by AMPA as well as for the inhibitory effects of ethanol.

There was a small component of AMPA receptor-mediated response which could be mediated by voltage-gated $\text{Ca}^{2+}$ channels. The possibility that the inhibitory effects of ethanol
may be related to the inhibition of these Ca\(^{2+}\) channel-dependent component, could, however, be excluded by the experiments with nifedipine and ethanol alone and in combination, in which additive inhibitory effects were demonstrated.

It is well established that interneurons have powerful regulatory roles in controlling the activity of complex neuronal circuits in diverse brain structures (Ross and Soltesz, 2001). As a cellular consequence of acute ethanol exposure (e.g. a decreased AMPA and NMDA receptor function), the excitability of interneurons can be reduced and consequently the inhibitory control function to pyramidal neurons is diminished. However, detailed studies have shown that various neuronal cell populations as well as multiple ligand- or voltage-gated ion channels are affected by ethanol and may contribute to the known hypnotic or intoxicating effects of this substance at higher concentrations (Fairgolnd et al., 1998; Little, 1999). Therefore, the significance of the observed decrease in Ca\(^{2+}\) transients for neuronal function is still a matter of debate. Finally, although there is now compelling evidence for the fact that excitatory amino acid receptors are involved in major actions of ethanol, the key question for a specific site of interaction at the NMDA or AMPA/kainate receptor channels remains hitherto unanswered (Woodward, 1999).

Acknowledgements — We thank Mrs H. Sobottka for skilful technical assistance in preparing the cortical cell cultures. This study was supported by the Deutsche Forschungsgemeinschaft (II 209/2–2).

REFERENCES


