ELSEVIER

European Journal of Pharmacology 394 (2000) 41–45

ejp

www.elsevier.nl/locate/ejphar

Short communication

Inhibition by chloral hydrate and trichloroethanol of AMPA-induced Ca^{2+} influx in rat cultured cortical neurones

Wolfgang Fischer *, Clemens Allgaier, Peter Illes

Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Universität Leipzig, Härtelstr. 16-18, D-04107 Leipzig, Germany

Received 20 December 1999; received in revised form 17 February 2000; accepted 22 February 2000

Abstract

The effects of chloral hydrate and its main metabolite 2,2,2-trichloroethanol were investigated on the (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-induced rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured non-pyramidal cortical neurones of rats by using single-cell fura-2 microfluorimetry. AMPA elicited a concentration-dependent effect that peaked at 300 μM (EC_{50}, 7.5 μM). Responses to AMPA (30 μM) were markedly inhibited by superfusion with chloral hydrate (IC_{50}, 4.5 mM) or trichloroethanol (IC_{50}, 0.9 mM). By contrast, ethanol (100 mM) caused only slight inhibition. In conclusion, the results demonstrate that chloral hydrate and especially its metabolite trichloroethanol, inhibit the AMPA-induced rise of $[Ca^{2+}]_i$ by depressing the entry of Ca^2 into cortical neurones via the AMPA receptor-channel.

Keywords: AMPA receptor; Cortical neurone, cultured; Chloral hydrate; Trichloroethanol; Ca^{2+} concentration, intracellular; Fura-2 microfluorimetry

1. Introduction

Chloral hydrate has been used clinically and experimentally as a sedative-hypnotic drug since its introduction by Liebreich in 1869 (see Haddad et al., 1998). It is well accepted that chloral hydrate is rapidly metabolized to 2,2,2-trichloroethanol, the main metabolite, which persists in the brain and body for hours and is believed to be responsible for the characteristic central depressant effects (Marshall and Owens, 1954; Breimer, 1977). However, the cellular mechanisms of action of these compounds are not yet clearly understood. In the last decade, evidence has been accumulated that trichloroethanol and related substances enhance $GABA_A$ receptor mediated Cl^- currents in various neuronal preparations (Weight et al., 1992; Lovinger et al., 1993; Peoples and Weight, 1994). Some authors also reported inhibitory effects of trichloroethanol on excitatory amino acid receptor-activated ionic currents (Weight et al., 1992); *N*-methyl-D-aspartate (NMDA)- or kainate-induced currents in hippocampal neurones were inhibited by trichloroethanol in the low millimolar range (Peoples and Weight, 1998). Investigations from our laboratory demonstrated an inhibitory action of trichloroethanol on NMDA-induced increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat mesencephalic and cortical neurones (Scheibler et al., 1999).

In the present study, the concentration-dependent effects of chloralhydrate and trichloroethanol (in comparison with ethanol itself) on (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-induced increases in $[Ca^{2+}]_i$ were investigated in primary cultures of rat cortical neurones using fura-2 microfluorimetry. Some of the results have been communicated in abstract form (Fischer et al., 1999).

2. Materials and methods

2.1. Cortical cell cultures

Neuronal cultures were prepared from the cerebral cortex of 16-day-old rat embryos (Wistar strain) as previously described (Allgaier et al., 1999). Briefly, minced cortical tissue was dissociated enzymatically (0.25% trypsin) and by mechanical trituration. Finally, isolated neurones

* Corresponding author. Tel.: +49-341-972-4603; fax: +49-341-972-4609.

E-mail address: fisw@medizin.uni-leipzig.de (W. Fischer).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00160-6

(150.000 cells/cm^2) were seeded on glass coverslips precoated with poly-L-lysine and cultured in 35-mm dishes with Dulbecco's modified Eagle's medium/Nutrient F12 (1:1 mixture; Gibco BRL), supplemented with 20% foetal heat-inactivated calf serum, 2 mM L-glutamine, 36 mM D-glucose, 50 μg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. In order to prevent glial proliferation, cytosine-β-D-arabinofuranoside (10 μM, Sigma) was added for 24 h after 6 days of culturing.

2.2. $[Ca^{2+}]_i$ measurement

Cortical neurones (10–15 days in vitro) were loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2 acetoxymethyl ester (5 μM; Sigma) at 37°C for 30 min in culture medium. Then, the cells were incubated for an additional 30 min in a fura-2-free superfusion medium (composition in mM: NaCl 133, KCl 4.8, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.3, HEPES 10, D-glucose 10; pH 7.4 adjusted with NaOH) at room temperature to remove extracellular traces of the dye. Subsequently, the coverslips were mounted into the open bottom of a perfusion chamber (250 μl), placed on the stage of an inverted microscope with epifluorescence optics (Diaphot 200, Nikon). Throughout the experiments, cells were continuously superfused (at 0.8 ml/min) with drug-free or drug-containing medium by means of a roller pump. Fluorescence ratio measurements were made on morphologically identified non-pyramidal neurones (multipolar, medium-sized) with a dual-wavelength spectrometer (alternating excitation at 340 and 380 nm). Fura-2 fluorescence was measured (over somatic regions) at 510/520 nm by a microscope photometer attached to a photomultiplier detection system (Ratiomaster System; PTI). Complete data acquisition, presentation and analysis were performed computer-controlled by using commercially available software (PTI, FeliX, Vers. 1.1). Calibration of $[Ca^{2+}]_i$ was performed with 10 μM ionomycin and 25 mM EGTA according to Grynkiewicz et al. (1985).

2.3. Application of drugs and data analysis

In order to construct concentration–response curves, AMPA (0.3–300 μM; Sigma) was applied at up to three different concentrations to the same neuron in random order, for 60 s and with an interval of 10 min. The concentration causing 50% of the maximum effect at 300 μM was termed EC_{50}. A submaximal concentration of AMPA (30 μM) was applied three times (S_1, S_2, S_3) for 60 s every 10 min. A Ca^{2+}-free medium (plus 1 mM EGTA), 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-3-methylcarbamoyl-2,3-benzodiazepine (GYKI 53655; gift of Dr. L. Harsing, Institute for Drug Research, Budapest, Hungary), 1,2,3,4,-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX), tetrodotoxin, nifedipine, ω-conotoxin MVIIC, chloral hydrate, trichloroethanol (all Sigma), D(−)-2-amino-5-phosphonopentanoic acid (AP-5; RBI), and ethanol (Mallinchrodt Baker) were superfused 10 min before and during S_2. The AMPA-induced rise of $[Ca^{2+}]_i$ was defined as the peak increase in the Δ fluorescence ratio 340/380 nm, i.e. the fluorescence ratio in response to AMPA minus the basal fluorescence ratio. Drug effects were evaluated as the percentage change of the AMPA-induced signal at S_2 vs. the signal at S_1. The difference between the AMPA-induced signals at S_1 and S_2 was assessed for statistical significance by the paired Student's *t*-test. In the absence of additional drugs, the AMPA (30 μM)-induced rise in $[Ca^{2+}]_i$ was reproducible ($1.9 \pm 0.5\%$ decrease, $n = 10$ cells; $P > 0.05$). The concentration causing 50% inhibition of the maximum possible effect was termed IC_{50}. The calculations of the EC_{50} and IC_{50} values were made with 3- or 4-parameter logistic functions (SigmaPlot, Vers. 5.0; SPSS). All data are expressed as means ± S.E.M. of *n* determinations.

3. Results

AMPA (0.3–300 μM) caused a concentration-dependent increase of $[Ca^{2+}]_i$ in about 80% of the non-pyramidal multipolar cortical neurones studied (maximal effect at 300 μM; EC_{50} value 7.5 ± 1.9 μM; $n = 6$–40 cells per concentration). AMPA (30 μM) was chosen for further experiments. This concentration induced a mean peak increase of Δ fluorescence ratio of 1.01 ± 0.05 which corresponds to an increase of somatic $[Ca^{2+}]_i$ from basal 81.5 ± 5.7 to 484.2 ± 30.5 nM ($n = 6$ cells). The response to AMPA was almost abolished in a Ca^{2+}-free medium with 1 mM EGTA ($99.3 \pm 0.3\%$ inhibition; $n = 6$; $P < 0.001$) or in the presence of the selective AMPA receptor antagonists GYKI 53655 (10 μM) ($98.9 \pm 0.6\%$; $n = 8$; $P < 0.001$) as well as NBQX (30 μM) ($96.6 \pm 1.2\%$; $n = 6$; $P < 0.001$). The effect of AMPA was insensitive to tetrodotoxin (0.3 μM) ($0.5 \pm 3.9\%$; $n = 6$; $P > 0.05$) and various Ca^{2+} channel blockers such as nifedipine (10 μM) ($8.5 \pm 6.5\%$; $n = 6$; $P > 0.05$) and ω-conotoxin MVIIC (0.1 μM) ($4.8 \pm 2.5\%$; $n = 7$; $P > 0.05$) as well as the NMDA receptor antagonist AP-5 (60 μM) ($0.9 \pm 1.6\%$; $n = 6$; $P > 0.05$).

Superfusion with the sedative-hypnotic drugs chloral hydrate (0.3–10 mM) or trichloroethanol (0.1–10 mM) concentration-dependently diminished the amplitude of AMPA-induced $[Ca^{2+}]_i$ rises (see Fig. 1 for representative tracings). Chloral hydrate (1 mM) caused a suppression of about 25% of the $[Ca^{2+}]_i$ signal (Fig. 1A). Up to this concentration, the washout was nearly reversible. Trichloroethanol appeared to be more potent than its parent compound. The average inhibition of the AMPA re-

sponse by trichloroethanol (1 mM) was greater than 50% (Fig. 1B). Higher concentrations ($\geq$ 3 mM) decreased also the latency and rise time of the $[Ca^{2+}]_i$ signals. It is noteworthy that trichloroethanol at 10 mM caused striking morphological changes and signs of cellular injury (granulated, clumped cytoplasm, darkening and visualization of the nuclei). The basal Ca^{2+} level of the neurones irreversibly increased to ratios of 1.5 and higher.

The concentration–response curves revealed a higher potency for trichloroethanol (IC_{50}, 0.9 $\pm$ 0.1 mM) than for chloral hydrate (IC_{50}, 4.5 $\pm$ 1.8 mM) (Fig. 2). On the other hand, ethanol up to 100 mM only slightly diminished the AMPA-induced $[Ca^{2+}]_i$ increase (maximally by 18.1 $\pm$ 1.4%; $n = 8$; $P < 0.01$) (Figs. 1C and 2). The $[Ca^{2+}]_i$ signals were fully reversible upon the washout of ethanol.

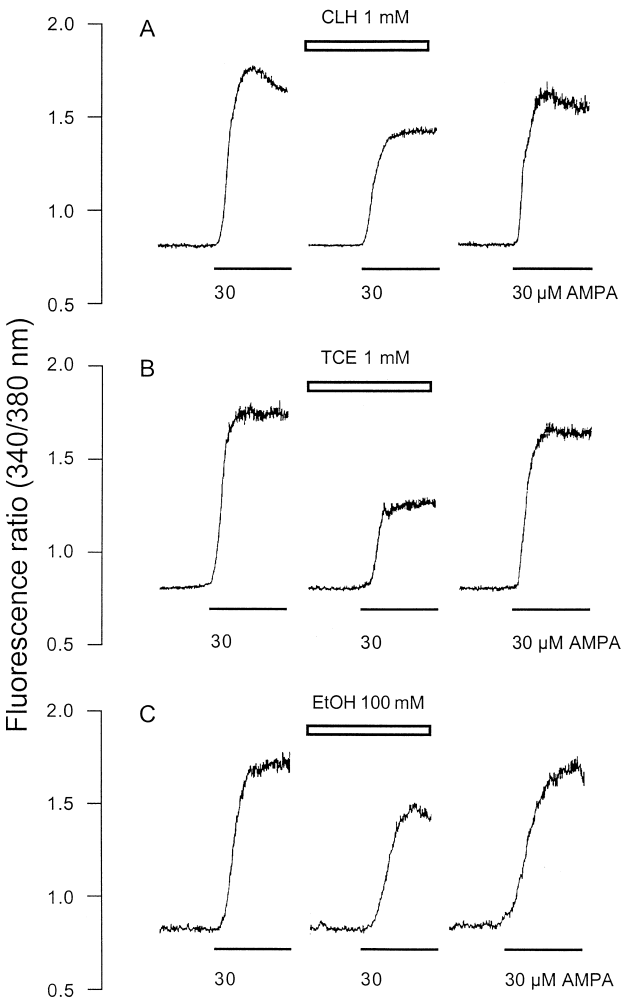

Fig. 1. Effects of chloral hydrate, trichloroethanol and ethanol on the AMPA-induced increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured non-pyramidal cortical neurones of rats. Ratios of the fluorescence intensities at 340 vs. 380 nm were measured by using fura-2 microfluorimetry. Representative tracings from single-cell experiments are shown. AMPA (30 µM) was superfused three times for 60 s every 10 min; the sedative-hypnotic drugs were applied 10 min before and during the second AMPA application. Subsequently, recovery was tested 10 min after washout with drug-free superfusion medium. Effects of chloral hydrate (CLH; A) trichloroethanol (TCE; B) and ethanol (ETOH; C) on responses to AMPA.

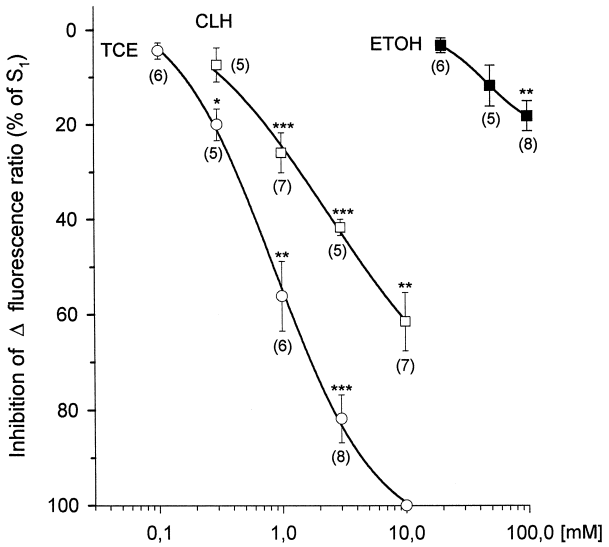

Fig. 2. Concentration-dependent inhibition by chloral hydrate, trichloroethanol and ethanol of the AMPA-induced increase of $[Ca^{2+}]_i$ in cultured rat cortical neurones. AMPA (30 µM) was superfused three times for 60 s every 10 min (S_1, S_2, S_3); the sedative-hypnotic drugs were superfused 10 min before and during the second AMPA application. Data are shown as means $\pm$ S.E.M., expressed as a percentage of the control response at S_1; the number of cells tested are indicated in parentheses. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (paired Student's *t*-test).

4. Discussion

Primary cultures of cortical neurones were prepared from 16-day-old rat embryos. Previous immunocytochemical studies showed that approximately 80–90% of the neurones were GABA as well parvalbumin positive (W. Fischer and H. Franke, unpublished results). In agreement with data in the literature (Hertz et al., 1989), it can be concluded that under our conditions, the cultures are highly enriched in GABAergic interneurones.

The present study investigated the inhibitory activity of the sedative-hypnotic drug chloral hydrate and its active metabolite trichloroethanol on the AMPA-induced increase of $[Ca^{2+}]_i$ in non-pyramidal multipolar cortical neurones. It was shown that the effect of AMPA was due to the entry of Ca^{2+} via the receptor channel itself (see also Carriedo et al., 1998). The opening of voltage-sensitive Ca^{2+} or Na^{+} channels as a consequence of the AMPA-induced depolarization was not causally related to the rise of $[Ca^{2+}]_i$ as shown by experiments with nifedipine, ω-conotoxin MVIIC and tetrodotoxin. The AMPA effect was abolished by the selective antagonists NBQX and GYKI

53655 (Bleakman and Lodge, 1998), but not by the NMDA receptor antagonist AP-5.

From the clinical point of view, the inhibition by trichloroethanol appears to be more important than that of its parent compound, since chloral hydrate is rapidly and quantitatively converted to trichloroethanol in vivo. It is possible, however, that chloral hydrate effects per se contribute to the known central depressant activity (Marshall and Owens, 1954; Grüner et al., 1973). The inhibitory potency of trichloroethanol on the AMPA (IC_{50}, 0.9 mM; present study) and NMDA (IC_{50}, 2.8 mM; Scheibler et al., 1999) receptor-mediated $[Ca^{2+}]_i$ rise of cultured cortical neurones was almost identical. Thus, inhibition of excitatory amino acid-induced responses was not selective for any of the subtypes of ionotropic glutamate receptors. Interestingly, it should be noted that the inhibition of NMDA and/or AMPA receptor function may be one important mechanism, whereby various general anaesthetics act (Carlà and Moroni, 1992).

In rat hippocampal neurones, trichloroethanol potentiates the current response to GABA at $GABA_A$ receptors (Weight et al., 1992; Peoples and Weight, 1994) and inhibits the current response to NMDA and kainate at their respective receptors (Peoples and Weight, 1998). In addition, trichloroethanol has been shown to facilitate 5-HT_3 receptor-mediated currents in nodose ganglion neurones (Lovinger and Zhou, 1993). Therefore, interactions with various neurotransmitter-operated ion channels seem to be involved in the pharmacological effects of these drugs.

In humans, it appears that after the therapeutic administration of chloral hydrate (hypnotic doses of 1–2 g p.o.) the peak plasma concentration of trichloroethanol reaches levels of ≥ 100 μM (Haddad et al., 1998; see also Marshall and Owens, 1954; Breimer, 1977). However, human brain concentrations of the lipophilic metabolite trichloroethanol are unknown. Plasma concentrations of trichloroethanol in the low millimolar range, which may markedly interact with excitatory or inhibitory amino acid receptors (see above), were observed in laboratory animals after the application of higher doses of chloral hydrate (Krieglstein and Stock, 1973; Owen and Taberner, 1980) or in humans after fatal chloral hydrate poisoning (Levine et al., 1985).

In conclusion, the present results demonstrate that chloral hydrate and its active metabolite trichloroethanol concentration dependently inhibit AMPA-induced increase of $[Ca^{2+}]_i$ in cultured rat cortical neurones. This effect is due to a suppression of Ca^{2+} entry via the AMPA receptor channel and may contribute to the central depressant as well as toxic properties of these drugs.

Acknowledgements

The authors wish to thank Mrs. H. Sobottka for excellent technical assistance in preparing the cortical cell cultures and Dr. P. Scheibler for methodological advice in single-cell fura-2 microfluorimetry. This study was supported by the Bundesministerium für Bildung, Forschung und Technologie, Biologische und Psychosoziale Faktoren von Drogenmißbrauch und Drogenabhängigkeit (01EB-9709/1 and 01EB9804/6).

References

Allgaier, C., Scheibler, P., Müller, D., Feuerstein, T.J., Illes, P., 1999. NMDA receptor characterization and subunit expression in rat cultured mesencephalic neurones. Br. J. Pharmacol. 126, 121–130.

Bleakman, D., Lodge, D., 1998. Neuropharmacology of AMPA and kainate receptors. Neuropharmacology 37, 1187–1204.

Breimer, D.D., 1977. Clinical pharmacokinetics of hypnotics. Clin. Pharmacokinet. 2, 93–109.

Carlà, V., Moroni, F., 1992. General anaesthetics inhibit the responses induced by glutamate receptor agonists in the mouse cortex. Neurosci. Lett. 146, 21–24.

Carriedo, S.G., Yin, H.Z., Sensi, S.L., Weiss, J.H., 1998. Rapid Ca^{2+} entry through Ca^{2+}-permeable AMPA/kainate channels triggers marked intracellular Ca^{2+} rises and consequent oxygen radical production. J. Neurosci. 18, 7727–7738.

Fischer, W., Franke, H., Scheibler, P., Allgaier, C., Illes, P., 1999. Characterization of Ca^{2+} responses to AMPA in cultured rat cortical neurones by fura-2 microfluorimetry. Naunyn-Schmiedeberg's Arch. Pharmacol. 359, R89, (Suppl.).

Grüner, J., Krieglstein, J., Rieger, H., 1973. Comparison of the effects of chloral hydrate and trichloroethanol on the EEG of the isolated perfused rat brain. Naunyn-Schmiedeberg's Arch. Pharmacol. 277, 333–348.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Haddad, L.M., Shannon, M.W., Winchester, J.F, 1998. In: Clinical Management of Poisoning and Drug Overdose. 3rd edn. Saunders, Philadelphia, PA, pp. 581–608.

Hertz, E., Yu, A.C.H., Hertz, L., Juurlink, B.H.J., Schousboe, A., 1989. Preparation of primary cultures of mouse cortical neurons. In: Shahar, A., de Vellis, J., Vernadakis, A., Haber, B. (Eds.), A Dissection and Tissue Culture Manual of the Nervous System. Wiley-List, New York, NY, pp. 183–186.

Krieglstein, J., Stock, R., 1973. Comparative study of the effects of chloral hydrate and trichloroethanol on cerebral metabolism. Naunyn-Schmiedeberg's Arch. Pharmacol. 277, 323–332.

Levine, B., Park, J., Smith, T.D., Caplan, Y.H., 1985. Chloral hydrate: unusual high concentrations in a fatal overdose. J. Anal. Toxicol. 9, 232–233.

Lovinger, D.M., Zhou, Q., 1993. Trichloroethanol potentiation of 5-hydroxytryptamine$_3$ receptor-mediated ion current in nodose ganglion neurons from the adult rat. J. Pharmacol. Exp. Ther. 265, 771–776.

Lovinger, D.M., Zimmerman, S.A., Levitin, M., Jones, M.V., Harrison, N.L., 1993. Trichloroethanol potentiates synaptic transmission mediated by γ-aminobutyric acid$_A$ receptors in hippocampal neurons. J. Pharmacol. Exp. Ther. 264, 1097–1103.

Marshall, E.K. Jr., Owens, A.H. Jr., 1954. Absorption, excretion and metabolic fate of chloral hydrate and trichloroethanol. Bull. Johns Hopkins Hosp. 95, 1–18.

Owen, B.E., Taberner, P.V., 1980. Studies on the hypnotic effects of chloral hydrate and ethanol and their metabolism in vivo and in vitro. Biochem. Pharmacol. 29, 3011–3016.

Peoples, R.W., Weight, F.F., 1994. Trichloroethanol potentiation of γ-aminobutyric acid-activated chloride current in mouse hippocampal neurones. Br. J. Pharmacol. 113, 555–563.

Peoples, R.W., Weight, F.F., 1998. Inhibition of excitatory amino acid-

activated currents by trichloroethanol and trifluoroethanol in mouse hippocampal neurones. Br. J. Pharmacol. 124, 1159–1164.

Scheibler, P., Kronfeld, A., Illes, P., Allgaier, C., 1999. Trichloroethanol impairs NMDA receptor function in rat mesencephalic and cortical neurones. Eur. J. Pharmacol. 366, R1–R2.

Weight, F.F., Aguayo, L.G., White, G., Lovinger, D.M., Peoples, R.W., 1992. GABA- and glutamate-gated ion channels as molecular sites of alcohol and anesthetic action. In: Biggio, G., Concas, A., Costa, E. (Eds.), GABAergic Synaptic Transmission. Raven Press, New York, NY, pp. 335–347.