Volume 247, number 1, 61–67 FEB 06989 April 1989

Ethanol potentiates and blocks NMDA-activated single-channel currents in rat hippocampal pyramidal cells

Maria Teresa R. Lima-Landman* and Edson X. Albuquerque*+

*Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201, USA and +Laboratory of Molecular Pharmacology, Institute of Biophysics, Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941, Brazil

Received 8 February 1989

Single-channel currents activated by *N*-methyl-D-aspartate (NMDA) were characterized using the outside-out patch clamp technique in cultured hippocampal cells from the rat. Several conductance states were observed, and the main one of 47 pS was further analyzed for channel lifetime and frequency. Open times decreased with hyperpolarization of the membrane. In view of recent evidence linking NMDA receptors to central nervous system processes such as learning and memory and ethanol (EtOH) tolerance, the effects of EtOH (0.01–1%, v/v, or ≃1.74–174 mM) were studied in this preparation. Two effects of EtOH could be discerned: (i) at low concentrations (1.74–8.65 mM) an increase in the probability of opening (p_{open}) of the NMDA-activated channel currents, without change in the mean channel open time, and (ii) at higher concentrations (86.5–174 mM) a decrease in p_{open} with a concomitant decrease in the mean open time. It is suggested that EtOH, even at rather low concentrations, may affect important brain functions.

Methyl-D-aspartate, *N*-; Single-channel current; Ethanol effect; (Hippocampus)

1. INTRODUCTION

The mechanisms by which EtOH affects the central nervous system remain to be fully elucidated. Behavioral studies using animals acutely and chronically intoxicated with ethanol (EtOH) have suggested that the stimulatory effect produced by low doses of EtOH appears to be mediated by the activation of central dopaminergic transmission while an inhibitory effect is related to central GABAergic transmission [1,2]. More detailed analysis indicated that EtOH enhances the openings of $GABA_A$ receptor-gated chloride channels [3–6]. It has also been shown that stimulation of dopaminergic activity could be due to a depression of the activity of the GABAergic mechanisms [2], thus relating GABA to both phases of the EtOH activity. Indeed, a number of authors postulated a role of the GABA system in the development of EtOH tolerance and dependence [4,7–9].

Although the actions of EtOH have been extensively studied on GABAergic systems, few data exist on the effects of EtOH on excitatory amino acid systems [10,11]. *N*-Methyl-D-aspartate (NMDA) receptor/channel complex has been implicated in some of the most important functions of the CNS such as learning and memory [12,13], and these processes have been recently correlated to the well-described phenomenon of tolerance after EtOH administration [14].

In preliminary studies we observed that at low concentrations EtOH had an effect on the NMDA-activated single currents [15]. The objective of this work was to study the action of EtOH on the NMDA-activated single-channel currents in rat hippocampal cells, and also to characterize further the NMDA receptor-ion channel.

Correspondence address: M.T.R. Lima-Landman, Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201, USA

Abbreviations: FDUR, fluoro-2′-deoxyuridine; NMDA, *N*-methyl-D-aspartate; p_{open}, probability of opening

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

We have observed that EtOH at low concentrations activated and blocked the NMDA receptor-ion channel. These findings suggest that even at low levels EtOH affects important brain functions.

2. MATERIALS AND METHODS

2.1. *Tissue culture*

The method utilized was basically the one described by Aracava et al. [16]. Briefly, female rats (Sprague-Dawley, 16–18 days of gestation) were killed by cervical dislocation and the fetuses placed in cold physiological solution. The cerebral hemispheres were isolated and the hippocampi dissected, minced and incubated with trypsin (0.25%) for 30 min at 35.5°C. After the incubation period, the medium was changed to modified Eagle's medium (MEM, Gibco) with 10% horse serum, 10% fetal calf serum, glutamine (2 mM) and DNase (40 μg/ml). The neurons were dissociated by trituration with a Pasteur pipette and plated in a final concentration of 350000 cells/ml in Petri dishes previously covered with astrocytes isolated from DUB mice [17]. 24 h later the culture medium was changed to MEM plus 10% horse serum and glutamine (2 mM). 1 week after plating, FDUR (53 μM) was added to avoid background cell proliferation, and after 24 h the medium was changed to MEM plus 10% horse serum and glutamine (2 mM). The medium was changed twice a week. The hippocampal cultures were composed principally of pyramidal cells, as the granular cells were not present at this stage of development. For recording channel activity, 7–21-day-old cultures were used.

2.2. *Patch-clamp technique*

The recordings of single-channel currents were made using the patch-clamp technique [18] in the outside-out configuration. The microelectrode resistance ranged between 2 and 7 MΩ. Recordings were made at room temperature (19–22°C). The external solution had the following composition (mM): 165 NaCl, 5 KCl, 2 $CaCl_2$, 5 Hepes (pH 7.3), 310 mosm plus 0.3 μM TTX. The internal solution was composed of (mM): 80 CsCl, 80 CsF, 10 CsEGTA, 10 Hepes, pH 7.3; 320 mosm [19]. An LM-EPC 7 patch-clamp system (List Electronic, FRG) was used to record the single-channel currents. The data were stored on FM tape (Racal 4DS), filtered at 3 kHz and digitized at 12.5 kHz. The IPROC-2 program was used for analysis of current amplitude and channel open and closed times. Open probability p_{open} was calculated as the sum of all open durations during a recording divided by the record length. Because the IPROC-2 program (Axon Instruments, Burlingame, CA) excludes openings to a multiple conductance level from open time histograms an attempt was made to correct the total open time for these events; the number of events invalidated because of openings to a multiple conductance level was multiplied by the mean open time, and this value was added to the sum of the open times. Unfortunately, this method still provides an underestimation, since two or more openings to a multiple level would be counted as only one if they occurred within a single open event. The histograms of channel open and closed times were fitted using the NFITS program (Axon Instruments).

3. RESULTS

3.1. *NMDA-activated single-channel currents*

NMDA-activated single-channel currents recorded from cultured hippocampal pyramidal neurons are shown in fig.1. Amplitudes were voltage-dependent, and the current-voltage plot yielded slope conductance values of 47 and 30 pS for the two highest conductance states (fig.1). The mean channel open time under control conditions decreased with hyperpolarization (fig.2). At a holding potential of -70 mV these channels had a mean channel open time of 1.39 ± 0.36 ms ($n = 14$ patches), and this value remained constant regardless of the concentration of NMDA used (2.5–10 μM). The decrease in mean open time with hyperpolarization occurred together with an increase in the number of flickers, and the duration of the flickers showed some voltage dependence. At -70 mV the second component of the closed time distribution had a mean of 0.80 ± 0.16 ms ($n = 10$ patches), while at -120 mV this value was increased to 1.44 ± 0.26 ms ($n = 5$ patches). Frequency of openings varied from patch to patch, but did not change during the time of recording, i.e. no desensitization pattern was seen even at the highest (50 μM) concentrations of NMDA tested. However, at concentrations ≥ 250 μM some degree of desensitization was noted (Ramoa and Albuquerque, unpublished).

3.2. *EtOH effects on NMDA-activated channel currents*

NMDA concentrations were selected based on the frequency of channel opening in the patch. The data are presented as percentage of the values recorded after a stabilization period of at least 1 min after adding NMDA. Control recordings were first made in the presence of NMDA alone and then solutions containing test concentrations of EtOH (0.01–1%, v/v, or $\simeq$1.74–174 mM) were added to the bath, allowing at least 1 min to achieve homogeneous dilution of EtOH in the bath. Several concentrations of EtOH could be tested on a single patch, allowing at least 2 min for recording at each concentration. When it was possible, recording was also made after washing. The effects of the low concentrations of EtOH (1.74–8.65 mM) tested were not uniform. Usually there was an increase in the frequency of openings

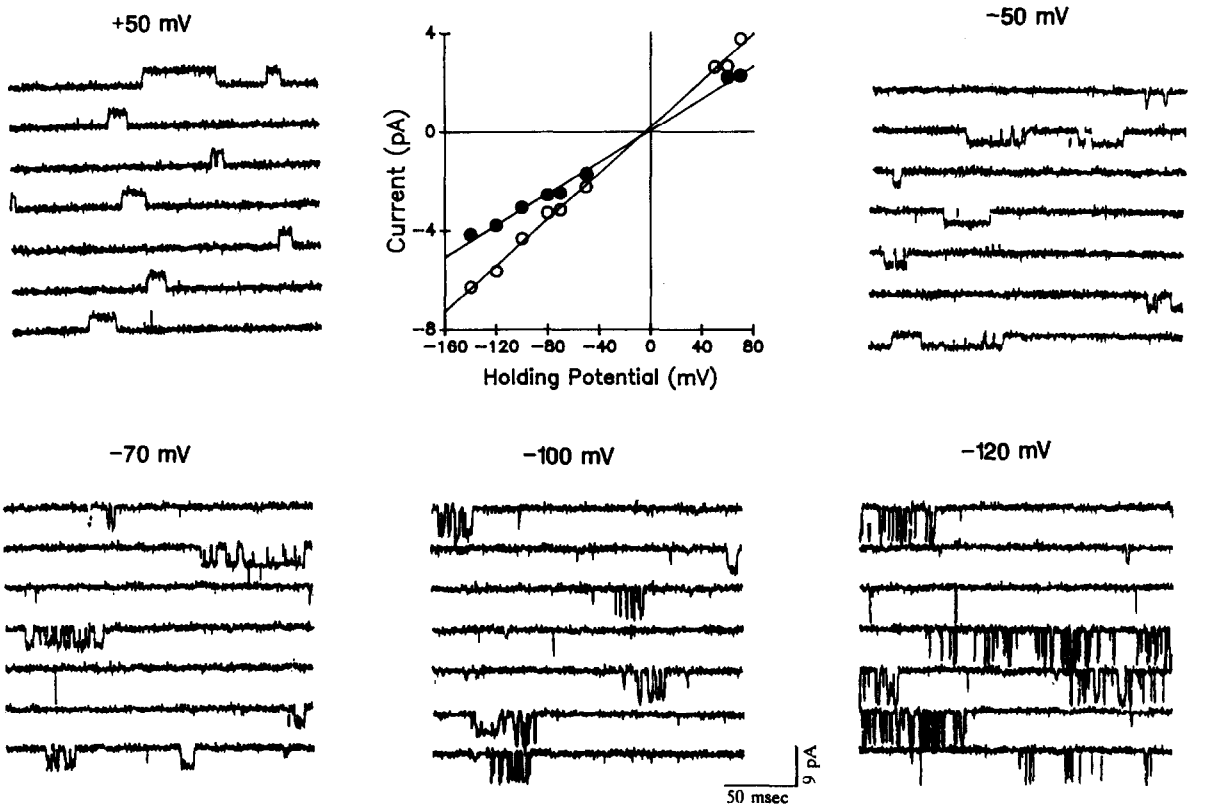

Fig.1. Samples of NMDA-activated currents at various holding potentials. Amplitude of NMDA (10 μM)-activated single channel currents is also plotted vs membrane potential. Channel current amplitude varied linearly with the holding potential, and two predominant conductances, a lower one of 30.0 ± 2.7 pS (●) and a higher one of 47.1 ± 2.1 pS (○) were observed. Data were obtained from 4 patches. The standard error is below the resolution of the graph (<0.05% of the mean).

reflected as a 48% increase in p_{open}, but in some experiments instead of an increase in the probability of channel opening, a decrease of this parameter (21%) was observed (fig.3). When the concentration of EtOH was increased 10 times (86.5–174 mM), a depressant effect of EtOH on the frequency of openings of the NMDA-activated channel currents was seen. The p_{open} decreased to 50% of the control values (fig.3). This same magnitude of decrease was observed whether the recording was made immediately after obtaining the control NMDA recordings or at the end of a series of concentrations of EtOH.

The discrimination between the effects of low and high concentration of EtOH was also seen on the mean open time. The mean open time of the NMDA-activated currents was not affected by 1.74, 8.65 and 17.4 mM of EtOH, but it was de-

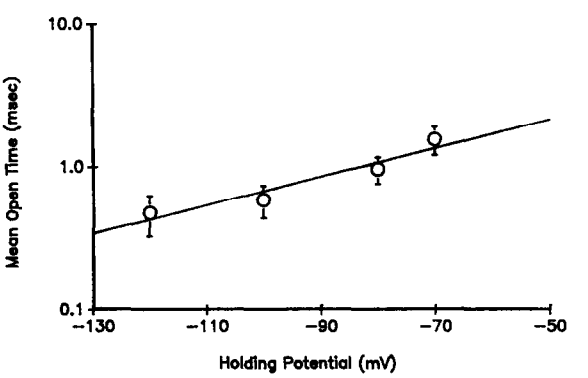

Fig.2. Mean open time of NMDA (10 μM)-activated single-channel currents. The logarithms of values for channel open time in the range of potentials studied were linearly related to holding potential, with a positive slope and an e-fold change in open time per 45 mV.

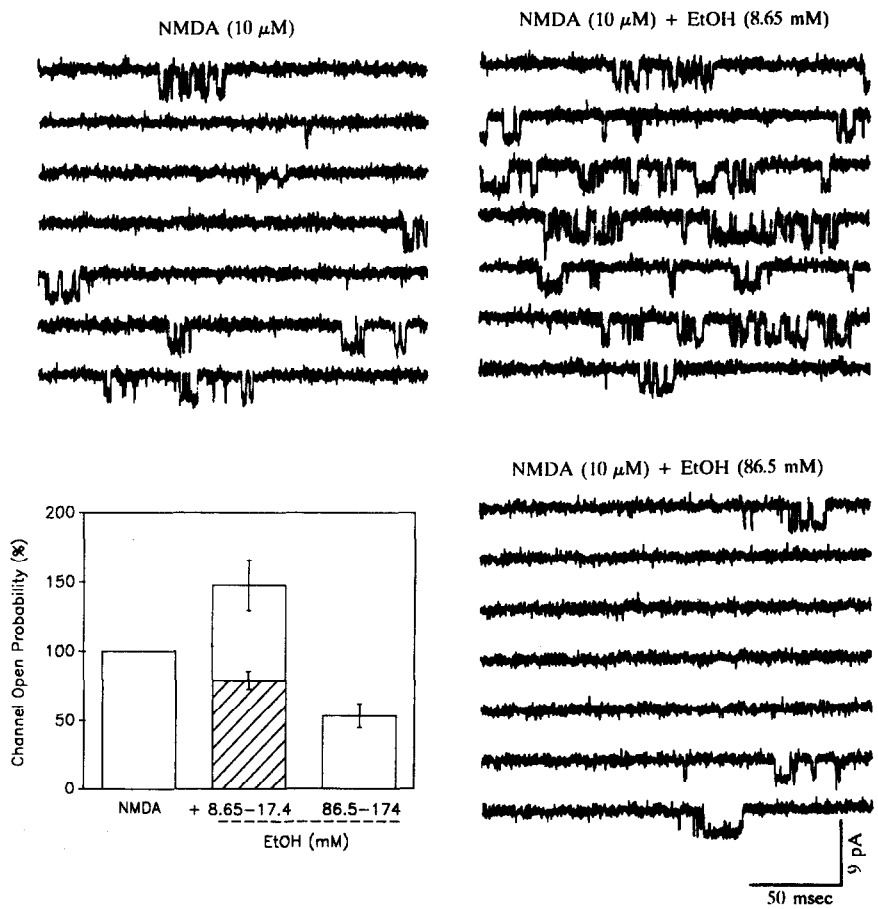

Fig.3. Samples of NMDA (10 μM)-activated single-channel currents. Recordings are from one outside-out patch. The graph shows the probability of opening (p_{open}) is plotted in the inset. The increase in p_{open} was seen in 5 out of 7 patches. The decrease in p_{open} with the low concentrations was seen in 2 out of 7 patches. Because more than one recording was made in the two patches which showed no increase, the total number of observations was four. The 50% decrease observed after the higher concentrations of EtOH was seen in all patches studied. Holding potential −70 mV, NMDA concentration for the inset ranged from 2.5 to 10 μM.

creased to 85% and 58% of the control values in the presence of 86.5 and 174 mM of EtOH, respectively (figs 4 and 5).

These effects of EtOH on the frequency and open time of NMDA-activated channel currents were partly recovered upon washing.

4. DISCUSSION

Single-channel currents activated by 2.5–10 μM NMDA showed at least three conductance levels, the higher ones having conductance of 47 and 30 pS. Similar values have been reported previously [19–21]. The currents flickered markedly at hyperpolarized potentials, resulting in a decrease in open time with hyperpolarization in the range of potentials tested. It is possible that these are characteristics of the NMDA-activated channels, themselves, but we cannot discard the possibility that this behavior could be due to an open channel blockade. NMDA could be blocking its own channels, or we could be seeing a blockade by Mg^{2+} [22]. Values for mean open time for a given holding potential were constant at the different concentrations of NMDA used in these experiments, arguing against the possibility that NMDA is acting as an open channel blocker. Regarding Mg^{2+}, even though solutions were

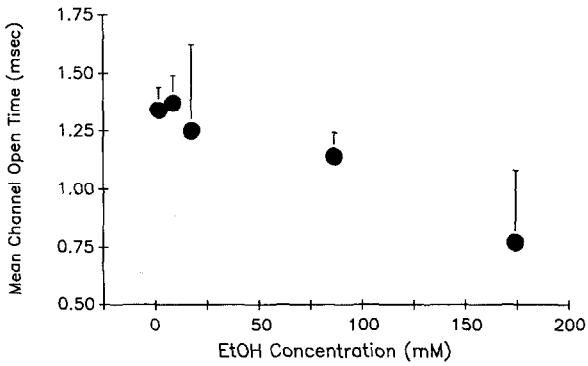

Fig.4. Effect of EtOH on the mean open time of NMDA-activated single-channel currents. Data are from 3–5 patches. Holding potential −80 mV.

nominally Mg^{2+}-free, and we used the perfusion system for some patches to protect against contamination by Mg^{2+} liberated by the cells per se, a small amount of this cation may still be available that could block the NMDA channels [22]. Indeed, the voltage dependence that we observed for the flicker durations seems to be in agreement with blockade by a charged ion. In contrast, Howe et al. [23] recording from cerebellar granule cells of the rat found no voltage dependence of duration and number of flickers, leading these authors to conclude that the NMDA-activated channels were not being blocked by a charged molecule. Preliminary experiments using EDTA (5 mM) to chelate Mg^{2+} showed no change in flickering behavior of the NMDA-activated currents (Aracava, personal communication). More experiments are needed to clarify this issue.

We have shown that EtOH, in the range of concentrations tested (0.01–1%, v/v, or ≃1.74–174 mM), affected the frequency of openings and the mean open time of the NMDA-activated single-channel currents. While at 8.65–17.4 mM EtOH, the majority of patches showed an increase in frequency, in two out of seven patches studied we saw a decrease in frequency. We can only speculate that more than one population of NMDA receptors that exhibit conductance of 47 pS may exist, only one of which responds with an increase in frequency to EtOH. Since the facilitatory effect lasted for 5–10 min in the presence of EtOH, it is unlikely that an increase in frequency was missed in these patches during the 1 min equilibration before recording.

Effects of EtOH in rat cerebellar Purkinje cells have been shown to be concentration dependent, i.e. low concentrations of the drug (4.4 mM) increase the firing rate and the regularity of the cerebellar activity, and high concentration (55 mM) depress the activity [24,25]. In Purkinje neurons also, chronic exposure to EtOH (19.5 mM) results in a decrease in the responsiveness of these neurons to glutamate and, when the treatment is done during development, it alters the spontaneous activity of the neurons without impairing their morphological differentiation [11]. Similar to our results, the effects seen after acute administration of EtOH [10] were reversible, in contrast to results seen after chronic administration, when the EtOH effects persist after removal of the agent [11].

The concentration of ethanol required to cause acute intoxication is ≥30 mM and at a concentra-

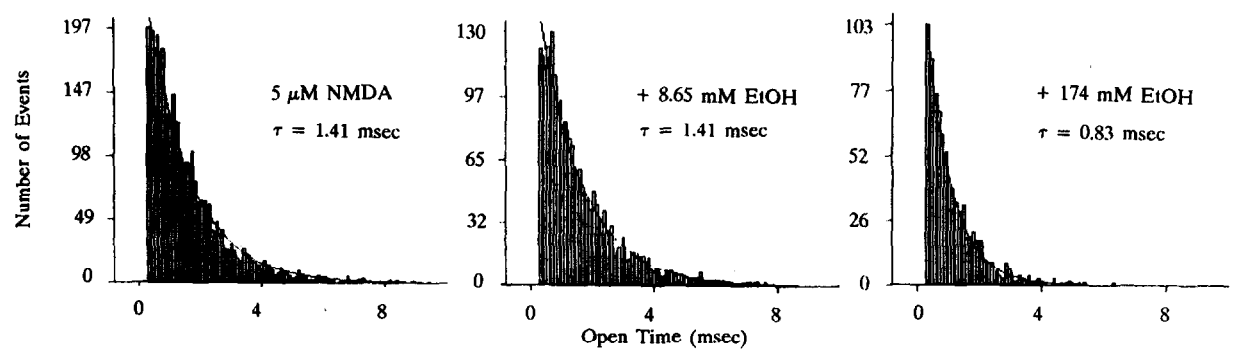

Fig.5. Open time histograms of NMDA-activated single-channel currents recorded in the presence of 5 μM NMDA plus 8.65 or 174 mM EtOH. Holding potential −80 mV.

tion of ≥100 mM it has lethal effects [26]. Suzdak et al. [6] showed that ≥20 mM of EtOH stimulate Cl^- uptake via GABA receptor-coupled chloride ion channels in cerebral cortical synaptosomes, but subintoxicating concentrations (≤10 mM) potentiate both muscimol- and pentobarbital-stimulated Cl^- uptake. In the present work the range of concentrations of EtOH used was 1.74–174 mM, a relatively low level when compared to the values seen in the literature [6,26]. The effects seen on the NMDA-activated channel currents at such a low concentration may not be due to unspecific effects of EtOH on the membrane lipids themselves. MacCreery and Hunt [27] showed that the ability of EtOH to induce ataxia in rats correlates with its membrane/buffer partition coefficient and membrane disordering properties suggesting that the behavioral effects of EtOH were not induced by specific binding to a receptor. The range of concentrations of EtOH used in the present work and the reversibility of the effects after washing suggest that these EtOH effects could be specifically related to an action on the NMDA receptor ion channel complex.

Recently, some homology has been proposed between different chemically gated channels [28]; between some regions of the $GABA_A$ receptor and the nicotinic receptor this homology reached 62% [29]. Structural as well as pharmacological and biochemical similarities have been reported [28,30]. These findings are not restricted to the GABA/nicotinic receptor; resemblances were also seen between the nicotinic receptor and the glycine receptor [31]. These observations led to the hypothesis that the ligand-gated channels are derived from a common ancestor, the chloride/bicarbonate channel [32]. Because the action of EtOH is well established in the $GABA_A$ receptor ion channel, the effects of this agent on the NMDA-activated channel current could reinforce the concept of the super-family of receptors.

The increase in the frequency of openings of the NMDA-activated single channel currents seen at a low concentration of EtOH could be the result of an increase in the affinity of NMDA for its binding site. On the other hand this effect could result from the stimulation of presynaptically located sites causing the release of endogenous glutamate, consequently increasing the frequency of openings of the NMDA-activated channel. However, such an effect could result in receptor desensitization [33,34], a phenomenon not observed in this study with NMDA.

The decrease in the mean open time of the NMDA-activated single currents seen at higher concentrations of EtOH could lead to a marked decrease in the activity of some central areas related to these excitatory amino acids. Thus, the ability of EtOH to increase the excitability of the glutamate receptor, seen with low concentrations of the agent, strongly suggests a stimulatory effect of the agent on brain activity mediated by glutamate, whose implication in the physiopathological function remains to be elucidated.

In summary, EtOH, over the range of concentrations tested, seems to have two effects: at low concentrations it facilitates the activation of the NMDA receptor/ion channel without changing the mean open time, but at higher concentrations it decreases the frequency of openings and the channel lifetime. These actions of EtOH seen at rather low concentrations suggest that NMDA receptors could be intimately involved in the lack of consciousness, coordination and convulsions seen after EtOH administration as well as in the development of alcohol tolerance.

Acknowledgements: The authors wish to thank Mrs Barbara Marrow and Ms Mabel Zelle for technical and computer assistance. This work was supported by US Army Medical Research and Development Command Contract DAMD17-88-C-8119 and by Financiadora de Estudos e Projetos (FINEP), Brazil.

REFERENCES

[1] Hunt, W.A. (1985) in: Alcohol and Biological Membranes, pp.103–129, Guilford Press, New York, NY.
[2] Mereu, G. and Gessa, G.L. (1985) Brain Res. 360, 325–330.
[3] Allan, A.M. and Harris, R.A. (1985) Pharmacologist 27, 125.
[4] Allan, A.M. and Harris, R.A. (1987) Pharmacol. Biochem. Behav. 27, 665–670.
[5] Allan, A.M., Spuhler, K.P. and Harris, R.A. (1988) J. Pharmacol. Exp. Ther. 244, 866–870.
[6] Suzdak, P.D., Schwartz, R.D., Skolnick, P. and Paul, S.M. (1986) Proc. Natl. Acad. Sci. USA 83, 4071–4075.
[7] Carlen, P.L. and Corrigall, W.A. (1980) Neurosci. Lett. 17, 95–100.
[8] Ticku, M.K. and Burch, T. (1980) J. Neurochem. 34, 417–423.

[9] Nordberg, A., Wahlström, G. and Eriksson, G. (1985) Eur. J. Pharmacol. 115, 301–304.
[10] Franklin, C.L. and Gruol, D.L. (1987) Brain Res. 416, 205–218.
[11] Yool, A.J. and Gruol, D.L. (1987) Brain Res. 420, 205–219.
[12] Morris, R.G.M., Anderson, E., Lynch, G.S. and Baudry, M. (1986) Nature 319, 774–776.
[13] Collingridge, G.L. (1985) Trends Pharmacol. Sci. 6, 407–411.
[14] Tabakoff, B. and Hoffman, P.L. (1988) Alcoholism: Clin. Exp. Res. 12, 184–186.
[15] Lima-Landman, M.T. and Albuquerque, E.X. (1988) Soc. Neurosci. Abstr. 14, 96.
[16] Aracava, Y., Deshpande, S.S., Swanson, K.L., Rapoport, H., Wonnacott, S., Lunt, G. and Albuquerque, E.X. (1987) FEBS Lett. 222, 63–70.
[17] Brookes, J. and Yarowsky, P.J. (1985) J. Neurochem. 44, 473–479.
[18] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[19] Jahr, C.E. and Stevens, C.F. (1987) Nature 325, 522–525.
[20] Ascher, P., Pregestovski, P. and Nowak, L. (1988) J. Physiol. 399, 209–226.
[21] Cull-Candy, S.G. and Usowicz, M.M. (1987) Nature 325, 525–528.
[22] Ascher, P. and Nowak, L. (1988) J. Physiol. 399, 247–266.
[23] Howe, J.R., Colquhoun, D. and Cull-Candy, S.G. (1988) Proc. R. Soc. Lond. B 233, 407–422.
[24] Seil, F.J., Leiman, A.L., Herman, M.M. and Fisk, R.A. (1977) Exp. Neurol. 55, 390–404.
[25] Chu, N. (1983) Int. J. Neurosci. 21, 265–278.
[26] Paul, S.M. and Meyer, R.E. (1987) Psychopharmacol. Bull. 23, 445–451.
[27] McCreery, M.J. and Hunt, W.A. (1978) Neuropharmacology 17, 451–461.
[28] Schwartz, R. and Mindlin, M. (1988) J. Pharmacol. Exp. Ther. 244, 963–970.
[29] Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A., Seeburg, P.H. and Barnard, E.A. (1987) Nature 328, 221–227.
[30] Schwartz, R. (1988) Biochem. Pharmacol. 18, 3369–3375.
[31] Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E.D. and Betz, H. (1987) Nature 328, 215–220.
[32] Kosower, E.M. (1988) FEBS Lett. 231, 5–10.
[33] Katz, B. and Thesleff, F. (1957) J. Physiol. 138, 63–80.
[34] Sakmann, B., Patlak, J. and Neher, E. (1980) Nature 286, 71–73.