Biochemical Pharmacology, Vol. 29, pp. 1903–1908.

0006-2952/80/0701-1903 $02.00/0

EFFECTS OF ACUTE AND CHRONIC ETHANOL ADMINISTRATION ON WHOLE MOUSE BRAIN SYNAPTOSOMAL CALCIUM INFLUX

MITCHELL B. FRIEDMAN, CARLTON K. ERICKSON and STEVEN W. LESLIE
College of Pharmacy, Department of Pharmacology, The University of Texas at Austin, Austin, TX 78712, U.S.A.

(*Received* 30 *August* 1979; *accepted* 5 *January* 1980)

Abstract—Whole brain synaptosomes, isolated from pair-fed acute *in vitro* (sucrose–Sustacal diet for 10 days), acute *in vivo* [4.5 g/kg, 20% (w/v) i.p. ethanol pretreatment] and chronic *in vivo* (ethanol–Sustacal diet for 10 days) female, Swiss–Webster mice, were challenged *in vitro* with ethanol (80 mM, final concentration) in either an incubation medium (12-min exposure), a depolarizing medium (2-min exposure with 74 mM KCl) or a nondepolarizing medium (2-min exposure with 5 mM KCl). Depolarizing and nondepolarizing media also contained $^{45}Ca^{2+}$ (2 μCi/μmole). The results showed (1) a significant enhancement of $^{45}Ca^{2+}$ influx when synaptosomes isolated from acute *in vitro* and acute *in vivo* mice groups were challenged *in vitro* by ethanol (80 mM) in the depolarizing medium (74 mM KCl), (2) a significant enhancement by 80 mM ethanol of $^{45}Ca^{2+}$ accumulation by nondepolarized synaptosomes isolated from the acute *in vitro* mouse group, (3) a significant increase in $^{45}Ca^{2+}$ accumulation in synaptosomes from acute *in vivo* mice as compared with acute *in vitro* mice without an *in vitro* ethanol challenge, and (4) a significant decrease in $^{45}Ca^{2+}$ accumulation by synaptosomes isolated from chronic *in vivo* mice as compared to acute *in vitro* synaptosomes. The results presented here demonstrate the ability of ethanol to significantly increase calcium accumulation into whole brain synaptosomes and that tolerance to this phenomenon occurs in parallel with behavioral tolerance to the sedative action of ethanol. These ethanol-induced changes in calcium accumulation may be involved in the production of sedation and tolerance to sedation.

Although the use and abuse of ethanol have been present in every culture, the mechanisms by which it produces central nervous system depression are still shrouded in controversy. Much of this controversy centers upon the effects of ethanol on neurotransmitter activity in the central nervous system. Many studies have been undertaken to elucidate the changes in neurotransmitter activity subsequent to the administration of ethanol. Erickson and Graham [1] demonstrated with cortical collecting cups, the ability of ethanol to depress acetycholine release. Kalant *et al.* [2] also showed a decreased nondepolarized release of acetycholine in rat brain slices. In contrast, Richter and Werling [3] demonstrated an increased nondepolarized release af acetylcholine from brain slices in the presence of ethanol. Tabakoff [4] has recently reviewed the various conflicting reports concerning serotonin, dopamine and γ-aminobutyric acid release subsequent to both acute and chronic ethanol exposure. The apparent confusion in the literature concerning the actions of ethanol on neurotransmitter systems may be reduced somewhat when experimental variables, such as the concentrations of ethanol used and the time periods for assessing the effects of ethanol on neurotransmitter release, turnover, and synthesis, are examined in detail. Another important aspect of this problem is the biphasic nature of the behavioral effects of ethanol. An understanding of this may provide insight into the conflicting reports since, in the process of human intoxication, initial stimulation by ethanol is reversed, resulting in depression. It is reasonable to assume that the definition of a cellular mechanism which could explain these behaviors would provide significant information relating to the observed discrepancies in neurotransmitter activity.

Recent investigations in our laboratory demonstrated that calcium-mediated "stimulus–secretion coupling" is a locus of action for barbiturate-induced central nervous system depression [5–8]. The barbiturates inhibit potassium-stimulated calcium uptake into synaptosomes, and tolerance develops to this inhibition within the same time frame as behavioral tolerance. Such an inhibition of calcium uptake would then result in a decrease in neurotransmitter release subsequent to drug exposure [9]. Further examination of this phenomenon has shown that different brain areas are affected to varying degrees [8].

These results led to the present study involving ethanol and synaptosomal calcium influx to determine if ethanol shares a common site of action with the barbiturates in disrupting calcium influx and if biochemical events relating to behavioral tolerance could be observed.

MATERIALS AND METHODS

Female Swiss–Webster mice (18–25 g) were kept on a 12/12 hr light/dark cycle with food and water *ad lib.* before being randomly divided into three groups: acute *in vitro*, acute *in vivo* and chronic *in vivo*. Acute *in vitro* animals were pair-fed a sucrose–Sustacal diet along with the chronic *in vivo* animals which received an ethanol–Sustacal diet. The ethanol–Sustacal diet consisted of 315 ml of choco-

late Sustacal mixed with 53 ml of a 3.5 kcal/ml ethanol solution. This mixture was diluted to a final volume of 500 ml with distilled water. The sucrose–Sustacal diet was the same, substituting 53 ml of a 3.5 kcal/ml sucrose solution for the ethanol solution. Animals were kept on the diets for 10 days prior to the preparation of synaptosomes. At no time were the animals allowed to be without ethanol prior to decapitation. Blood levels were taken every morning by decapitating the animals and collecting 50 μl samples from ten animals chosen at random. Acute *in vivo* animals received a 4.5 g/kg i.p. dose of ethanol (20%, w/v) 45 min prior to decapitation.

Ethanol 'sleep-time' (loss of righting reflex, unconsciousness) was used to determine tolerance development in the chronic *in vivo* group. Animals were given a 4.5 g/kg i.p. dose of ethanol (20%, w/v) on days 2 and 9 of the diet regimen. Sleep-times were recorded as time from loss of righting reflex to time of regaining the righting reflex, which was defined as the ability to turn over from a supine position twice within 1 min.

Synaptosomes were prepared from whole brain by the method of Cotman [10]. Whole brains were homogenized using a Thomas size C homogenizing tube with a Teflon pestle at the lowest possible speed setting on a Sorvall Omni-mixer using ten up and down strokes. Centrifugation procedures were identical to those of Cotman [10]. The final synaptosomal pellet was resuspended in incubation medium to give a protein concentration of 0.6–0.8 mg/ml as determined by the method of Lowry *et al.* [11].

The accumulation of $^{45}Ca^{2+}$ by synaptosomes was studied as described by Leslie *et al.* [12]. A 0.5 ml aliquot of the synaptosomal preparation from each treatment group was added to 0.5 ml of incubation medium (NaCl, 136 mM; KCl, 5 mM; $CaCl_2 \cdot 2H_20$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) and allowed to equilibrate for 12 min at 30° in a Dubnoff metabolic shaker. Ethanol (80 mM, final concentration) was added to some of the incubating samples for 12 min to examine the effects of an *in vitro* ethanol challenge at this time interval. Loading of synaptosomes with $^{45}Ca^{2+}$ was initiated by the addition of depolarizing medium (KCl, 213 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) containing $^{45}Ca^{2+}$ (sp. act. of 2 μCi $^{45}Ca^{2+}$/μmole $^{40}Ca^{2+}$) and in some cases 80 mM ethanol (final concentration). This resulted in a 1.5 ml incubation volume containing 80 mM ethanol (final concentration) for ethanol samples and 74 mM KCl to depolarize the synaptosomal membrane [13]. After a 2 min incubation period, $^{45}Ca^{2+}$ loading was stopped by adding 5.0 ml of ice-cold EGTA* stopping solution (NaCl, 136 mM; KCl, 5 mM; $MgCl_2$, 1.3 mM; EGTA 3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid). Each sample was immediately filtered on a presoaked (250 mM KCl) 0.45 μm Millipore cellulose acetate filter using a Millipore microfiltration manifold. Each filter was washed with 10.0 ml of 0.32 M sucrose solution and placed in a scintillation vial containing Beckman Redy-Solv. Samples were counted using a Beckman LS-8000 scintillation counter. Counting efficiency was found to be approximately 72 per cent. Nondepolarized samples were handled in a similar manner except that, after the 12-min incubation period, 0.5 ml of incubation medium (5 mM KCl) containing $^{45}Ca^{2+}$ (2 μCi $^{45}Ca^{2+}$/μmole $^{40}Ca^{2+}$), and in some cases 80 mM ethanol (final concentration), was added. The exposure of synaptosomes to ethanol (80 mM, final concentration) in either the initial incubation medium or in the depolarizing or nondepolarizing media, allowed exposure times to ethanol to be either 12 or 2 min. Initial studies using concentrations of 40, 60 and 80 mM ethanol (final concentration) have provided support for a dose–response relationship for this effect (data not shown). Net influx of calcium across the synaptosomal membrane was calculated by the subtraction of the nondepolarized (5 mM KCl) values from the depolarized (74 mM KCl) values. This difference is expressed as Δ_k (potassium-induced change) and represents the calcium which is intimately involved in "stimulus–secretion coupling" events.

Blood alcohol values were determined using a Perkin–Elmer Multifract F40 gas chromatograph equipped for automatic head space analysis. Fifty-microliter samples of whole blood were placed in sealed glass containers and kept frozen until used. Samples were incubated in a 60° water bath for 15 min prior to automatic sample injection on a 20 m long by 2.7 mm (i.d.) diameter aluminum column with a carbowax 1500-chromosorb packing. Column temperature was 190°, carrier gas (N_2) flow was 30 ml/min, and retention time was 190 sec for ethanol. External standards were used for comparison.

RESULTS

Figure 1 represents the observed time course of the effect of ethanol on both depolarized (74 mM KCl) and nondepolarized (5 mM KCl) $^{45}Ca^{2+}$ accumulation by whole brain synaptosomes isolated from naive mice. In both the presence and the absence of 80 mM ethanol (final concentration), the plateau period for $^{45}Ca^{2+}$ influx appears to have begun at about a 2-min exposure to the depolarizing medium, and for this reason this time period was chosen for subsequent studies. It is apparent from these data that ethanol significantly increased calcium accumulation by both depolarized and nondepolarized synaptosomes as compared to control. The increase seen was most prominent up to the 4-min time period.

Figure 2 shows data from a series of experiments demonstrating the ability of ethanol to enhance calcium influx by both depolarized and nondepolarized synaptosomes subsequent to an *in vitro* ethanol challenge. In this set of experiments, ethanol significantly enhanced calcium accumulation in all samples, as compared to control, when a 2-min exposure was used. In agreement with data from Fig. 1, Fig. 2 shows that the same trend for increased $^{45}Ca^{2+}$ influx

* EGTA, ethyleneglycol-bis(beta-aminoethyl ether)-*N,N'*-tetra-acetic acid.

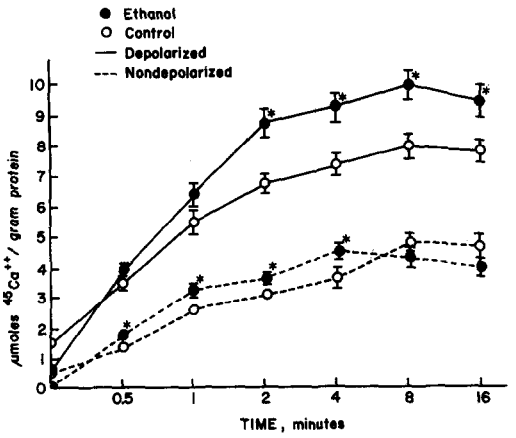

Fig. 1. Effects of ethanol (80 mM, final concentration) on the time course of $^{45}Ca^{2+}$ accumulation by depolarized (74 mM KCl) or nondepolarized (5 mM KCl) synaptosomes isolated from naive mice. Some synaptosomes were exposed to ethanol (80 mM) in either depolarizing or nondepolarizing media containing $^{45}Ca^{2+}$ for the times indicated, subsequent to a 12-min incubation at 30°, and compared to synaptosomes exposed to $^{45}Ca^{2+}$ in either depolarizing or nondepolarizing medium alone. Each point represents the mean ± S.E. of nine experiments (thirty-six animals). An asterisk (*) represents statistical significance as compared to the respective control value at $P < 0.05$ as determined by Students' *t*-test.

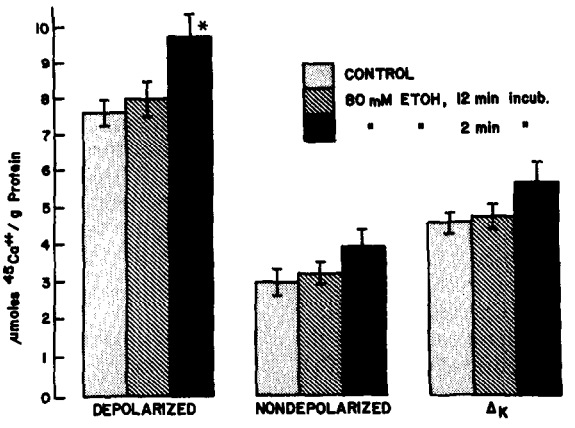

Fig. 3. Effects of an acute *in vitro* ethanol challenge (80 mM, final concentration) on $^{45}Ca^{2+}$ accumulation by depolarized (74 mM KCl) or nondepolarized (5 mM KCl) synaptosomes isolated from mice that received 4.5 g/kg of ethanol (20%, w/v) i.p. 45 min prior to synaptosomal isolation. Synaptosomes were challenged *in vitro* for either a 12-min or 2-min time period and compared with synaptosomes with no *in vitro* challenge. Each point represents the mean ± S.E. of nine experiments. An asterisk (*) represents significance at $P < 0.05$ as determined by analysis of variance and Student-Neuman-Keuls.

was present for the 12-min ethanol challenge but lacked a significant response as compared with control. The Δ_k showed a significant increase in synaptosomes treated with 80 mM ethanol for 2 min.

Figure 3 shows the effect of an *in vitro* ethanol challenge on $^{45}Ca^{2+}$ accumulation by synaptosomes isolated from animals which were decapitated 45 min subsequent to the administration of 4.5 g/kg of ethanol (20%, w/v) i.p. The results demonstrate that synaptosomes prepared from acutely treated animals showed a significant enhancement of depolarization-induced $^{45}Ca^{2+}$ accumulation as compared with the control in the 2-min exposure interval. No significant difference in either the nondepolarized or Δ_k values was seen as compared to control. This indicates that an acute adaptive response may have occurred to the *in vivo* ethanol challenge, when one recalls the data in Fig. 2.

Data shown in Fig. 4 represent the effects of ethanol (80 mM) *in vitro* on $^{45}Ca^{2+}$ accumulation by

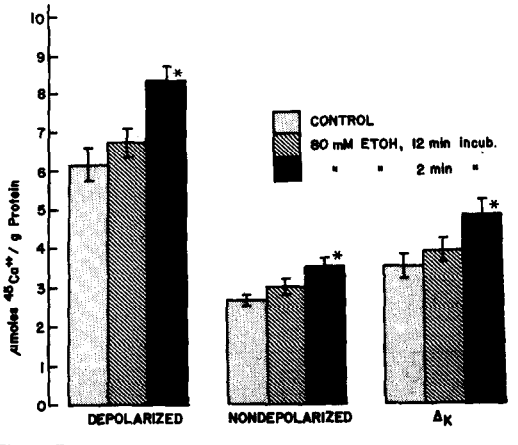

Fig. 2. Effects of an acute *in vitro* ethanol challenge (80 mM, final concentration) on $^{45}Ca^{2+}$ accumulation by depolarized (74 mM KCl) or nondepolarized (5 mM KCl) synaptosomes isolated from naive mice. Synaptosomes were challenged *in vitro* for either a 12-min or 2-min time period and compared with synaptosomes with no *in vitro* challenge. Each point represents the mean ± S.E. of thirteen experiments. An asterisk (*) represents significance at $P < 0.05$ as determined by analysis of variance and Student-Neuman-Keuls.

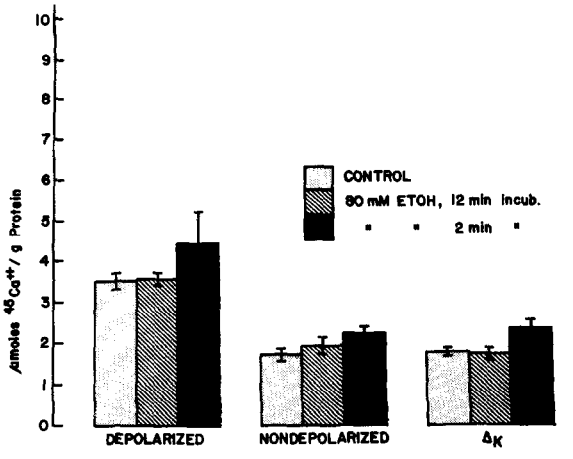

Fig. 4. Effects of an acute *in vitro* ethanol challenge (80 mM, final concentration) on $^{45}Ca^{2+}$ accumulation by depolarized (74 mM KCl) or nondepolarized (5 mM KCl) synaptosomes isolated from mice chronically exposed to ethanol by feeding them an ethanol–Sustacal diet for 10 days prior to synaptosomal isolation. Synaptosomes were challenged *in vitro* for either a 12-min or 2-min time period and compared with synaptosomes with no *in vitro* ethanol challenge. Each point represents the mean ± S.E. of nine experiments.

synaptosomes which were collected from mice chronically treated *in vivo* with an ethanol–Sustacal diet for 10 days. Although the same general trend in response to an *in vitro* ethanol challenge was observed (i.e. an increased calcium accumulation) as in the previous experiments, no significant difference was found between any of the treatment groups as compared with control. This finding indicates that chronic *in vivo* ethanol exposure resulted in an adaptation which manifested itself as a diminution in the ability of ethanol to enhance calcium influx across the nerve end.

Figure 5 illustrates the effects of *in vivo* ethanol administration on $^{45}Ca^{2+}$ accumulation by whole brain synaptosomes. Examination of these data shows that depolarized calcium accumulation was increased significantly in synaptosomes prepared from animals receiving an acute *in vivo* ethanol exposure. Conversely, chronic exposure *in vivo* to ethanol significantly decreased $^{45}Ca^{2+}$ accumulation (depolarized, nondepolarized and Δ_K) in synaptosomes prepared from animals chronically exposed to ethanol as compared to animals receiving no ethanol exposure. These data indicate that significant changes had occurred subsequent to *in vivo* ethanol administration which modified the responsiveness of synaptosomes even in the absence of an *in vitro* ethanol challenge.

Table 1 contains the ethanol sleep-time data obtained from animals treated chronically with an ethanol–Sustacal diet for 10 days. Animals served as their own controls, showing a significant reduction in ethanol sleep-time subsequent to a challenge dose of ethanol. Dietary consumption data are also shown indicating that average consumption per animal per day was 6.9 ml corresponding to 17.1 g/kg ethanol over a 24-hr period. Data from blood samples taken on the morning of days 2, 4, 6, 8 and 10 show that blood alcohol levels were maintained throughout the course of the study. Animals showed signs of intoxication, exhibiting ataxia and ptosis at various times during the 10-day exposure.

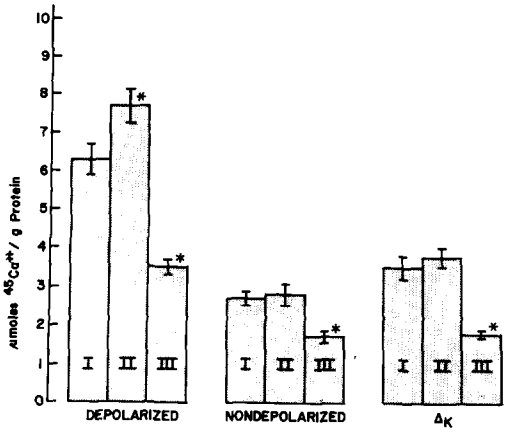

Fig. 5. Effects of *in vivo* ethanol administration on $^{45}Ca^{2+}$ accumulation by depolarized (74 mM KCl) or nondepolarized (5 mM KCl) synaptosomes isolated from mice which received no *in vitro* challenge. The data presented here are represented as 'control' data in Figs. 2, 3 and 4. Group I represents acute *in vitro* data, Group II represents acute *in vivo* data and Group III represents chronic *in vivo* data. An asterisk (*) represents significance at $P < 0.05$ as determined by analysis of variance and Student-Neuman-Keuls.

In a separate experiment, pair-fed sucrose control animals were compared with animals on plain laboratory chow *ad lib*. No significant difference occurred when synaptosomes prepared from the respective control groups were challenged with ethanol *in vitro* (data not shown).

DISCUSSION

That calcium is a necessity for stimulated release of neurotransmitters and other substances is well established [14]. Upon stimulation of the neuron, an inward calcium current is generated, with calcium flowing down a concentration gradient. Once inside, calcium couples the stimulus to a release of neurotransmitter via exocytosis.

Table 1. Sleep-time and blood alcohol concentration of rats treated chronically with ethanol

Group*	Average wt (g)	Average sleep-time ($\bar{X} \pm$ S.E.) (min)
Control	21.4	54.4 ± 4.4
Ethanol	18.2	20.4 ± 2.9†
Days on diet		**Blood alcohol conc‡ ($\bar{X} \pm$ S.E.) (mg/dl)**
2		60.0 ± 0.98
4		115.1 ± 30.3
6		98.6 ± 15.2
8		148.0 ± 28.7
10		88.4 ± 18.6

* Ethanol-treated animals consumed approximately 6.9 ml of the ethanol–Sustacal diet every 24 hr. This corresponds to a daily consumption of 17.1 g/kg of ethanol based on the mean weight of the animals. N = 19.

† Represents significance at $P < 0.01$ using Students' *t*-test (unpaired).

‡ Represents ethanol found in blood samples taken in the morning of the day listed. Analysis of variance of blood alcohol date showed no significant difference in blood alcohol values between days. N = 10.

Recent work has suggested that barbiturates may produce central nervous system sedation by inhibiting the depolarization-induced calcium influx across the nerve end [5, 7, 8, 13]. In addition, we have demonstrated that tolerance develops to this effect in parallel with the development of behavioral tolerance [5, 7, 8]. The results of the current study show that *in vitro* ethanol administration increased calcium accumulation by synaptosomes isolated from control mice. Chronic ethanol administration, however, resulted in a marked reduction in calcium accumulation by isolated synpatosomes. Thus, ethanol altered synaptosomal calcium accumulation, but its actions in this regard were essentially opposite to those observed with barbiturates.

Evidence exists for a depolarizing effect of ethanol on neuronal membranes. Eidelberg and Wooley [15] demonstrated a depolarizing effect of ethanol on spinal cord neurons. Sauerland *et al.* [16] have reported previously on the ability of ethanol to depolarize pre-synaptic trigeminal afferents. Gage [17], as well as others, has reported on the ability of ethanol to increase the frequency and amplitude of miniature end plate potentials (MEPP). Gallego [18] demonstrated the ability of ethanol to depolarize motoneurons and suggested that a depolarization blockade of sciatic nerve conduction was responsible for the action of ethanol in his preparation. In agreement with the idea that ethanol may produce a depolarization blockade, our results show that ethanol exposure facilitated synaptosomal calcium accumulation by resting synaptosomes for up to 4 min, after which time calcium accumulation in the presence of ethanol returned to control levels (Fig. 1). Also, Cooper and Dretchen [19] have examined the effects of ethanol on the neuromuscular junction and have proposed that the central effects of ethanol might be due to its excitatory pre-synaptic action. A recent report by Degani *et al.* [20] shows an increase in spontaneous norepinephrine release from the rat vas deferens in the presence of ethanol and calcium. They suggest that such an action by ethanol could conceivably decrease the 'signal to noise ratio' in the central nervous system, thereby interfering with neuronal communication.

In addition to the evidence of a peripheral depolarizing action of ethanol, reports concerning a central action, possibly indicating a central depolarizing response, exist as well. As noted earlier, Richter and Werling [3] showed an enhanced (although slight) nondepolarized release of acetylcholine in the presence of ethanol. Seeman and Lee [21] have previously reported an enhanced spontaneous release of dopamine from rat caudate synaptosomes in the presence of ethanol. That ethanol can increase calcium accumulation, perhaps via a direct depolarizing action on the neuronal membrane, suggests an enhanced release of neurotransmitter.

We have demonstrated that ethanol further enhances calcium uptake by potassium-stimulated synaptosomes as well (Fig. 1). The fact that ethanol enhanced calcium uptake in both stimulated and non-stimulated nerve ends may possibly explain the variety of results obtained by other workers who have measured neurotransmitter activity in the presence of ethanol. It appears from our data that both increased and decreased neurotransmitter release might be observed depending upon the time course of the experiment.

An apparent paradox is how one group of drugs can inhibit calcium accumulation (i.e. barbiturates) while the other enhances calcium accumulation (i.e. ethanol), yet both produce behavioral depression. Such a paradox in drug effects is not unique. A classic example of a similar situation is the neuromuscular blocking agents, one group of which prevents depolarization of the motor end plate while the other produces a depolarizing blockade; the two drugs have apparently opposite mechanisms but produce the same result.

Another aspect of the actions of ethanol relates to its ability to modify synaptosomal responsiveness subsequent to chronic *in vivo* ethanol administration and without an *in vitro* challenge, as depicted in Fig. 5. Chin and Goldstein [22] have previously shown the necessity for an *in vitro* ethanol challenge in order to see membrane fluidity changes in membrane fractions isolated from animals chronically exposed to ethanol. We have reported a similar finding in our previous work with the barbiturates [5, 7, 8]. The explanation for these differences remains elusive.

Consistent with the date presented here, Michaelis and Myers [23] have recently shown changes in calcium binding to a synaptosomal fraction from both acute *in vitro* and chronic *in vivo* ethanol challenge situations. This work shows that acute ethanol exposure, *in vitro*, leads to an increase in calcium binding followed by a decrease in calcium binding subsequent to chronic ethanol exposure. This decrease in calcium binding subsequent to chronic ethanol exposure was observed in the absence of an *in vitro* ethanol challenge. An *in vitro* challenge with ethanol produced no significant change in binding by the fraction isolated from chronically exposed animals, indicating an adaptive response at the level of the neuronal membrane.

Our data support the hypothesis that some of the pharmacologic effects of ethanol may be attributable to its ability to depolarize excitable membranes, as evidenced here by its ability to enhance calcium accumulation by synaptosomes. We have further shown that tolerance to this effect of ethanol on calcium influx develops within the same time frame as behavioral tolerance to the sedative effects of ethanol. Such an effect by ethanol may lead to either a depolarizing blockade or a decrease in the signal to noise ratio in the central nervous system, as has been suggested previously [20]. Further, Luqmani *et al.* [24] have recently shown that muscarinic receptors in synaptosomal membranes are greatly reduced in number following extended treatment with electrical pulses and veratridine, both of which serve as depolarizing stimuli. Thus, a complex picture of the actions of ethanol can be envisioned when one considers the implications of a depolarizing response coupled with such variables as differential effects due to the size of the neuron [25, 26] as well as differences in basal firing rates [27].

Acknowledgements—This work was supported by NIAAA grant AA03748-01. Salary for S. W. Leslie was supplied by NIAAA Research Scientist Development Award

AA00044–01. The authors would like to thank the Mead–Johnson Co. (Evansville, IN) for providing Sustacal for this study.

REFERENCES

1. C. K. Erickson and D. T. Graham, *J. Pharmac. exp. Ther.* **183**, 583 (1973).
2. H. Kalant, Y. Israel and M. A. Mahon, *Can. J. Physiol. Pharmac.* **45**, 172 (1967).
3. J. A. Richter and L. L. Werling, *J. Neurochem.* **32**, 935 (1979).
4. B. Tabakoff, in *Alcohol and Opiates: Neurochemical and Behavioral Mechanisms* (Ed. K. Blum), p. 21. Harcourt Brace Jovanovich, New York (1977).
5. M. G. Ondrusek, J. K. Belknap and S. W. Leslie, *Molec. Pharmac.* **15**, 386 (1979).
6. M. B. Friedman, R. R. Coleman and S. W. Leslie, *Life Sci.* **25**, 734 (1979).
7. S. W. Leslie, M. B. Friedman, R. E. Wilcox and S. V. Elrod, *Brain Res.*, **185**, 409 (1980).
8. S. V. Elrod and S. W. Leslie, *J. Pharmac. exp. Ther.* **212**, 131 (1980).
9. J. W. Haycock, W. B. Levy and C. W. Cotman, *Biochem. Pharmac.* **26**, 159 (1977).
10. C. W. Cotman, in *Methods of Enzymology* (Eds. L. Fleischer and L. Parker), p. 445. Academic Press, New York (1974).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. S. W. Leslie, S. V. Elrod, R. Coleman and J. K. Belknap, *Biochem. Pharmac.* **28**, 1440 (1979).
13. M. P. Blaustein and A. C. Ector, *Molec. Pharmac.* **11**, 369 (1975).
14. R. P. Rubin, *Pharmac. Rev.* **22**, 389 (1970).
15. E. Eidelberg and D. F. Wooley, *Archs int. Pharmacodyn. Thér.* **185**, 388 (1970).
16. E. K. Sauerland, N. Mizuno and R. M. Harper, *Expl. Neurol.* **27**, 476 (1970).
17. P. W. Gage, *J. Pharmac. exp. Ther.* **150**, 236 (1965).
18. A. Gallego, *J. cell comp. Physiol.* **31**, 97 (1948).
19. S. A. Cooper and K. L. Dretchen, *Eur. J. Pharmac.* **31**, 232 (1975).
20. N. C. Degani, E. M. Sellers and K. Kadzielawa, *J. Pharmac. exp. Ther.* **201**, 22 (1979).
21. P. Seeman and T. Lee, *J. Pharmac. exp. Ther.* **190**, 131 (1974).
22. J. H. Chin and D. B. Goldstein, *Science* **196**, 684 (1977).
23. E. K. Michaelis and S. L. Myers, *Biochem. Pharmac.* **28**, 2081 (1979).
24. Y. A. Luqmani, H. F. Bradford, N. J. M. Birdsall and E. C. Hulme, *Nature, Lond.* **277**, 481 (1979).
25. A. Staiman and P. Seeman, *J. Pharmac. exp. Ther.* **201**, 340 (1970).
26. A. Staiman and P. Seeman, *Can. J. Physiol. Pharmac.* **52**, 535 (1973).
27. E. Szabadi, C. M. Bradshaw and P. Bevan, *Brain Res.* **126**, 580 (1977).