Pharmacology Biochemistry & Behavior, Vol. 24, pp. 1659–1664, 1986. © Ankho International Inc. Printed in the U.S.A. 0091-3057/86 $3.00 + .00

Chronic Ethanol Administration Inhibits Calmodulin-Dependent Ca^{++} Uptake in Synaptosomal Membranes

DAVID H. ROSS[1]

Division of Molecular Pharmacology, Departments of Pharmacology and Psychiatry
The University of Texas Health Science Center, San Antonio, TX

Received 10 February 1984

ROSS, D. H. *Chronic ethanol administration inhibits calmodulin-dependent Ca^{++} uptake in synaptosomal membranes.* PHARMACOL BIOCHEM BEHAV 24(6) 1659–1664, 1986.—Chronic ethanol administration inhibits ATP-dependent Ca^{++} uptake in a preparation of synaptic membranes prepared from mice following 1, 4 and 7 days of ethanol exposure in a liquid diet. Addition of calmodulin (2.5 μg) to membranes from mice receiving the control diet produced a slight stimulation of ATP dependent Ca^{++} uptake. Membranes from ETOH treated mice exhibited reduced capacity to take up Ca^{++} in ATP-dependent fashion. When calmodulin was added to membranes isolated from mice receiving ETOH on Days 1, 4 and 7 ATP-dependent Ca^{++} uptake was significantly stimulated ($p<0.01$) compared to (1) ETOH treated membranes in absence of calmodulin, and (2) control membranes. Behavioral tolerance as estimated by bar holding technique was found to be 25,65 and 91 percent complete for Days 1, 4 and 7 respectively. These studies demonstrate that continued exposure of mice to ethanol via consumption of an ethanol containing liquid diet inhibits one of the mechanisms involving the cytosolic buffering of intracellular Ca^{++} in nerve terminals. This biochemical effect seen in parallel with the development of tolerance to ethanol impairment of bar holding suggests that increased cytosolic Ca^{++} may aid in central nervous system adaptation to ethanol.

Calmodulin Ethanol ATP-dependent Ca^{++}

CALMODULIN, an acidic Ca^{++} binding protein [3] has been implicated in a wide variety of Ca^{++}-dependent reactions, including adenylate cyclase, protein kinase, phosphorylation of membrane protein and Ca^{++} translocation by Ca^{++}/Mg^{++} ATPase [20,32]. Recent studies suggest that many of the calmodulin-dependent reactions in synaptic transmission are sensitive to a variety of CNS active drugs [12]. Among the various classes of drugs studied are agents such as neuroleptics and opiates, both of which are known to produce supersensitivity following chronic treatment. Behavioral supersensitivity following chronic haloperidol treatment has also been shown to occur in conjunction with increases in calmodulin content [10, 11, 15]. The content and bound/free distribution of calmodulin has been demonstrated to change following *in vitro* and *in vivo* drug administration. Similar results have recently been reported following the acute and chronic administration of both neuroleptic and opiate agonists [8, 12, 29, 38]. These studies are of interest since the mechanism of action of both these drugs has been studied with respect to second messenger systems such as Ca^{++} and protein phosphorylation.

The apparent changes in calmodulin content following drug administration may be reflective of ways in which calmodulin is bound to intracellular membrane sites. The binding of calmodulin has been shown to be Ca^{++}-dependent [36,37]; thus, any external perturbation of the membrane leading to changes in Ca^{++} content on the cytosolic membrane face may alter calmodulin content. Direct evidence to support this concept has been presented for neuroleptics [12, 14, 21] and opiates [18, 27, 28].

Ethanol has been reported to alter the content of Ca^{++} on synaptic membranes [26,31] and to alter the $^{45}Ca^{++}$ binding capacity in both erythrocyte [33] and synaptic membranes [16, 17, 31]. Chronic treatment with ethanol produced a decrease in $^{45}Ca^{++}$ binding capacity, which was the apparent result of an increase in CA^{++} content on the membrane [22,27].

Recent studies have demonstrated that calmodulin can enhance ATP-dependent Ca^{++} accumulation by synaptosomal membranes [32], which is believed to be one buffering mechanism for intracellular Ca^{++} [1, 2 32]. It was of interest, therefore, to study the effects of chronic ethanol administration on calmodulin activation of ATP-dependent Ca^{++} binding in mouse synaptosomal suspensions.

[1]Requests for reprints should be addressed to David H. Ross, Ph.D., Division of Molecular Pharmacology, Department of Pharmacology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7746.

METHOD

Preparation of Synaptosomes Membranes

Lysed synaptosomal suspensions were prepared, using the method of Cotman and Matthews [5], with the following modifications: Male ICR Swiss mice (25–30 g, Harlan Labs, Madison, WI) were sacrificed by decapitation and the brains quickly removed and washed in ice-cold saline to remove adhering blood vessels. Cerebellum and brainstem were discarded and a 10% homogenate was made of the remaining brain in 0.32 M sucrose, using a Teflon pestle-glass tissue homogenizer. The homogenate was centrifuged at 1000 × g for 10 min. The resulting S_1 supernatant was centrifuged at 17,500 × g for 20 min, resulting in a P_2 pellet. This pellet was gently resuspended in 0.32 M sucrose, layered over a 7.5–12% Ficoll gradient and centrifuged at 63,000 × g for 90 min. Synaptosomes were removed from the 7.5–12% interphase, washed free of Ficoll and resuspended in ice-cold hypotonic lysing buffer for 60 min. The buffer was composed of 20 mM Tris–0.5 mM dithioerythriotol, pH 8.5, at 25°C. Following this lysing procedure, the synaptosomes were pelleted by centrifugation at 38,000 × g for 20 min. The lysed synaptosomes were washed one time by this technique and the membranes resuspended to a protein concentration of 1 mg/ml as determined by the method of Lowry *et al.* For studies on calmodulin stimulation, the lysed synaptosomal membrane preparations were sequentially treated with EGTA, as described below, to remove endogenous calmodulin.

Preparation of Calmodulin-Deficient Synaptosomal Membranes

To remove calmodulin bound to the lysed synaptic membrane preparations, EGTA was used to sequentially treat the membrane preparations in a series of graded washes, according to the method of Sobue *et al.* [34]. Lysed synaptosomal membranes were resuspended in lysing buffer containing 2.5 mM EGTA and centrifuged at 38,000 × g for 20 min. The membrane pellet was resuspended in lysing buffer containing 1.0 mM EGTA for 60 min, pelleted and resuspended in lysing buffer containing 100 μM EGTA to a protein concentration of 1–1.5 mg/ml.

Assay of ATP-Dependent and -Independent Ca Uptake

Uptake of $^{45}Ca^{++}$ to synaptosomal plasma membranes in the presence and absence of ATP was determined by using the method of Ross and Cardenas [32]. Lysed synaptosomal membrane fractions, either native or EGTA-treated, were prepared as described above and incubated at 37°C under the following conditions: Membrane aliquots (100–150 μg) were resuspended in an incubation medium containing HEPES (20 mM), KCl (100 mM), $MgCl_2$ (250 μM), EGTA (100 μM), ouabain (1 mM) and $CaCl_2$, to achieve final $[Ca^{++}]_0$ of 0.1 to 5 μM, using calculations from Ross and Cardenas [32], with Ca^{++}-EGTA dissociation constants of 1.17×10^{-7} M^{-1} in a system of pH 7.1. The concentrations of ATP included throughout all reactions were 100 μM. Reactions were initiated by addition of $^{45}Ca^{++}$ (0.6 μCi) for 2 min followed by addition of ATP (100 μM). Net Ca^{++} accumulation was determined as the amount of Ca^{++} associated with membrane in the presence minus the amount of Ca^{++} in the absence of ATP and is expressed as ΔATP. Following the addition of ATP, the reaction was quenched 60 sec later with 3 ml of buffer identical to the above, except that ATP was replaced with 1.0 μM $CaCl_2$. The reaction mixture was filtered over Millipore filters (Hawp 0.45 μ, 24 mm) and washed three times with 4 ml of quench buffer. The filters were removed and allowed to dry, then counted in a Searle Isocap 300. The results of accumulation are expressed as nmoles/mg protein/min.

For experiments measuring calmodulin stimulation, membranes previously washed with EGTA were used in a fashion identical to that outlined above with the addition of calmodulin (2.5 μg) (purchased from Boehringer Mannheim) in HEPES buffer to each assay. This concentration of calmodulin was found to maximally stimulate the ATP-dependent Ca^{++} binding process with a K_m of 50–60 nM.

Chronic Administration of Ethanol

Mice were administered ethanol by the method of Ritzman and Tabakoff [30]. Mice were received from Harlan Labs one week prior to the initiation of chronic ethanol and during this time, maintained on lab chow ad lib until 24 hr prior to beginning the experiments with ethanol. During this 24 hr period, mice were fasted prior to the initiation of the liquid diet. Ethanol-treated mice and their yoked controls were individually caged and maintained under a constant temperature and humidity-controlled environment, with a 14/10 light/dark cycle. Mice received a 7% v/v ethanol solution in chocolate-flavored Sustacal fortified with 3 g/l solution of vitamin supplement. Control mice were paid-fed with Sustacal diets isocalorically supplemented with sucrose (96.8 g/l) and vitamins. Ad lib controls were also included to evaluate the nutritional adequacy of the Sustacal controls. No significant differences in weight between ad lib controls and Sustacal controls were seen. The weight and volume of liquid consumption of each animal was measured at 9:00 a.m. daily.

Behavioral Measurements

Development of behavioral tolerance to ethanol following chronic exposure was measured by the bar holding technique of Goldstein and Pal [13]. Animals were pretrained to hold on to a bar 0.3 inches in diameter, 24 inches above the table, for a minimum of 30 sec. Each mouse received a total of six trials. On Day zero (no ethanol), control and ethanolic mice were challenged with ethanol (1.5 g/kg of 20% w/v ethanol IP) and tested 5 min later for bar holding ability. The bar holding ability of each mouse was measured at 9:00 a.m. on Days 1, 4 and 7, to determine the degree of intoxication. Subsequent measurements on these days were done following an injection of 1.5 g/kg ethanol. Thus, tolerance was determined as the ability to hold the bar in the face of a constant challenge with ethanol.

Materials

KCl, toluene and trichloroacetic acid were obtained from MCB Manufacturing, Inc. $MgCl_2$ was purchased from Fisher Scientific and $CaCl_2$ from Baker Chemical Co. Ficoll, sucrose, EGTA, ouabain, HEPES, Trizma and DTE were purchased from Sigma. PPO and POPOP were supplied by Eastman Chemical Co. $^{45}Ca^{++}$ (4–10 Ci/g) was obtained from New England Nuclear. Ethanol diet solution was comprised of chocolate-flavored Sustacal obtained from Mead Johnson. Sucrose was purchased from MCB Manufacturing and the vitamin supplement from ICN.

TABLE 1

EFFECTS OF CHRONIC ETHANOL INTAKE ON BAR HOLDING RESPONSE

	Time on Bar (sec) (mean ± S.D.)		
	Day 1	Day 4	Day 7
Control			
Saline Injected Control	30.0 ± 1.8 (8)	30.0 ± 2.1 (8)	30.0 ± 2.3 (8)
Acute			
Sustacal (Pair Fed)	6.0 ± 3.1 (8)*	8.0 ± 2.3 (8)*	7.5 ± 1.9 (8)*
Chronic			
Sustacal-Ethanol	7.3 ± 2.0 (8)*	19.5 ± 2.4 (8)*	27.3 ± 1.6 (8)

Animals were tested following ethanol treatment at times shown in the following manner: Each mouse was tested 3 times in a five-minute period and the best trial of three was recorded. Each mouse had been previously tested under non-drug situations for training and accommodation purposes. Mice were tested for time on the bar following a 1.5 g/kg ethanol dose 20% w/v IP. Prior to this series of experiments, each mouse was tested to determine inability to stay on the bar 30 sec. These mice were discarded before the study began. One-way analysis of variance and Student-Newman-Keuls multiple comparison tests were used for statistical evaluation. Mean blood alcohol for all mice tested on Day 7 was 79.3 ± 22.5 mg %. Mean weight gain for the pair-fed mice for seven days was 0.4 ± 0.54 g, while the mean weight gain for the ethanolic mice was −0.99 ± 0.60 g. Numbers in parentheses indicate the number of mice in each group. The saline injected control group received only saline injections on Days 1, 4 and 7. The Sustacal group received 1.5 g/kg ethanol as a challenge on Days 1, 4 and 7. *Indicates significantly different from control (saline injected control) at $p<0.001$.

Statistics

A two tailed Student's *t*-test was used to calculate differences between control and treated groups.

Blood Alcohol Measurements

Blood samples were collected from the trunk of mice at sacrifice for blood ethanol analysis at 9:00 a.m. of the seventh day. Blood ethanol concentrations were determined by the head space technique of Wallace and Dahl previously outlined [9] using a Hewlett-Packard 5840 Model A gas chromatograph with a flame ionization detector and using isopropanol as an internal standard.

RESULTS

Assay of Tolerance Following Chronic Ethanol Administration

To correlate potential changes in the Ca^{++} buffering system with pharmacologically relevant effects of ethanol, we evaluated bar holding response in mice during various intervals following ethanol administration by liquid diet. Mice were tested for tolerance development following a fixed challenge dose of ethanol on Days 1, 4 and 7 (Table 1). Challenge following the first 24 hour period demonstrated a 25% degree of bar holding capacity while mice on Day 4 had developed 65% degree of bar holding capacity and by Day 7, mice demonstrated 91% capacity for bar holding compared to saline control. Pair-fed mice exhibited consistent bar holding capacity compared to saline control. Following ETOH challenge of 20%, 27% and 25% for Days 1, 4 and 7. This demonstrates the consistency of the ethanol challenge over seven days and dietary regimen using Sustacol diet for the ETOH vehicle. These results demonstrate a considerable degree of tolerance at seven days following a single dose of ethanol as a challenge. Blood alcohol levels in mice prior to challenge on day seven averaged 79.3 mg %.

Effects of Chronic Ethanol Administration on ATP-dependent Ca^{++} Uptake

Previous studies have reported that calmodulin can stimulate ATP-dependent Ca^{++} uptake into sarcoplasmic reticulum vesicles and smooth endoplasmic reticulum-like vesicles of lysed synaptosomal suspensions and synaptic membranes [28]. To characterize the membrane system used here it was necessary to study the effect of calmodulin addition *(in vitro)* on ATP-dependent Ca^{++} accumulation in membranes from control (pair fed) and ethanol treated mice. These results are illustrated in Table 2. Control membranes from mice receiving Sustacal for 1, 4 and 7 days were evaluated in absence of EGTA treatment for calmodulin stimulating capacity. Calmodulin was added to membrane preparations which had not been EGTA treated. As seen in Table 2, control ATP-dependent Ca^{++} uptake activity (ATP) remains relatively constant (3.61, 3.45, 3.42, nmoles/mg/min) from Days 1–7. These values agree well with control (ad lib fed mice) values previously reported [8]. Calmodulin addition (2.5 μg) produced a measurable activation (25%) over control indicating that not all calmodulin binding sites may be occupied under *in situ* conditions [32,33]. Calmodulin did not significantly alter ATP-independent Ca^{++} binding to membranes in agreement with previous reports [28] requiring ATP for Ca^{++} accumulation even in presence of optimal calmodulin.

Membranes from ethanol drinking mice were similarly evaluated for changes in ATP dependent and independent Ca^{++} uptake activity. As seen in Table 2, ethanol treatment did not significantly alter ATP-independent Ca^{++} binding but significantly decreased the ATP-dependent component as reflected by the decreased ATP at Days 1 and 7 (3.61 to 2.66; 3.42 to 2.34 nmoles/mg/min). To evaluate this in terms of calmodulin dependency, calmodulin was added *in vitro* to membranes from ETOH drinking mice on Days 1, 4 and 7. As seen in Table 2, calmodulin addition to ETOH treated membranes dramatically reverses the decreased ATP-

TABLE 2

EFFECTS OF ETHANOL AND CALMODULIN ON ATP-DEPENDENT Ca^{++} UPTAKE IN NATIVE MEMBRANES

	Ca^{++} Uptake (nmoles/mg/min) ± SEM		
	−ATP	+ATP	ΔATP
Control—Group 1 (6)			
Day 1	1.06 ± 0.13	4.67 ± 0.31	3.61
4	1.21 ± 0.17	4.66 ± 0.54	3.45
7	1.51 ± 0.46	4.96 ± 0.59	3.42
Control ± Calmodulin—Group 2 (6)			
Day 1	1.13 ± 0.16	5.49 ± 0.23	4.36*
4	1.41 ± 0.28	5.57 ± 0.49	4.16*
7	1.33 ± 0.16	5.79 ± 0.66	4.46*
ETOH—Group 3 (6)			
Day 1	1.10 ± 0.23	3.76 ± 0.21	2.66†
4	1.39 ± 0.23	4.16 ± 0.37	2.77†
7	1.31 ± 0.36	3.65 ± 0.18	2.34†
ETOH + Calmodulin—Group 4 (6)			
Day 1	0.97 ± 0.11	6.12 ± 0.38	5.15‡
4	1.20 ± 0.18	5.90 ± 0.92	4.70‡
7	1.71 ± 0.20	6.79 ± 0.78	5.08‡

*Significantly different compared to Control (Group 1) ($p<0.01$).
†Significantly different compared to Control (Group 1 and Group 4) ($p<0.01$).
‡Significantly different compared to Control (Group 1) and ETOH (Group 3) ($p<0.01$).
Synaptosomal membranes were obtained as outlined in the Method section. Calmodulin, obtained from commercial sources was added *in vitro* (2.5 μg) in appropriate experiments to control or treated membranes. Superscripts indicate significant differences at $p<0.01$. Values in parentheses indicate the number of mice in each group.

TABLE 3

EFFECTS OF ETHANOL AND CALMODULIN ON ATP-DEPENDENT Ca^{++} UPTAKE IN EGTA TREATED MEMBRANES

	Ca^{++} Uptake (nmoles/mg/min) ± SEM		
	−ATP	+ATP	ΔATP
Control (EGTA)—Group 1 (6)			
Day 1	0.96 ± 0.18	2.21 ± 0.13	1.25
4	1.08 ± 0.27	2.65 ± 0.77	1.57
7	1.31 ± 0.47	2.39 ± 0.35	1.08
Control (EGTA) + Calmodulin—Group 2 (6)			
Day 1	1.00 ± 0.15	5.85 ± 0.21	4.85*
4	0.97 ± 0.25	6.19 ± 0.47	5.22*
7	0.98 ± 0.25	6.97 ± 0.48	6.10*
ETOH (EGTA)—Group 3 (6)			
Day 1	1.49 ± 0.16	3.01 ± 0.19	1.52†
4	1.35 ± 0.16	2.45 ± 0.60	1.10†
7	0.95 ± 0.57	2.33 ± 0.41	1.57†
ETOH (EGTA) + Calmodulin—Group 4 (6)			
Day 1	1.75 ± 0.25	5.54 ± 0.17	3.79‡
4	0.90 ± 0.23	6.10 ± 0.86	6.70‡
7	0.89 ± 0.42	6.63 ± 0.34	5.74‡

*Significantly different compared to Control ($p<0.01$).
†Significantly different compared to Group 2 and Group 4 ($p<0.01$).
‡Significantly different compared to Control and ETOH ($p<0.01$).
Synaptosomal membranes were prepared with EGTA to remove endogenous calmodulin as outlined [34]. Calmodulin (2.5 μg) was added to the assay in appropriate experiments. Superscripts indicate the significant differences at $p<0.01$. Values in parentheses indicate the number of mice in each group.

dependent Ca^{++} uptake at each day tested. Little change is noted for ATP-independent Ca^{++} binding to membrane preparation. These studies suggest that ethanol exposure may alter the ability for calmodulin to interact with the membrane complex even in the presence of optimal Ca^{++} and ATP (Table 3). In order to examine this further, we performed studies where calmodulin was specifically removed from the membrane by EGTA treatment.

Effect of Ethanol and Calmodulin on ATP-Dependent Ca^{++} Uptake in EGTA-Treated Synaptosomal Membranes

To further define the requirement for calmodulin, we EGTA-treated the membranes from pair fed (Sustacal) and ethanol treated mice. In order to observe calmodulin-dependent ATP-dependent Ca^{++} uptake, endogenous calmodulin must be removed from the membrane [19, 24, 32]. As seen in Table 3, EGTA treatment of membranes from pair-fed mice (Group 1) resulted in substantial decrease in ATP-dependent Ca^{++} uptake in the presence of optimal Ca^{++}_{free} and ATP (100 μM), (ATP=1.25, 1.57 and 1.08 nmoles/mg/min) compared to Group 1, Table 2. EGTA treatment is believed to remove endogenously-bound calmodulin by disrupting the calmodulin·Ca^{++}·membrane complex [36,37]. To evaluate this, we performed a series of experiments adding calmodulin (2.5 μg) to the EGTA-treated membranes. As shown in Table 3, calmodulin stimulates the ATP-dependent Ca^{++} uptake system to optimum levels (see control + calmodulin Table 2). Ethanolic membranes were subjected to EGTA treatment and ATP-dependent and independent Ca^{++} uptake were determined. EGTA treatment did not alter ATP-independent Ca^{++} binding to membranes and produced no further decrease in ATP-dependent Ca^{++} uptake compared to EGTA-treated controls. This indicates that the Ca^{++} binding in absence of ATP is a pool unavailable to either EGTA or ETOH treatment. Also the studies indicate that ATP-dependent Ca^{++} uptake affected by EGTA and by ETOH treatment is the same pool since combined treatment produced no further decrease in ATP.

Again, addition of calmodulin to ethanol treated membranes which had been EGTA-treated, produced complete reactivation of ATP-dependent Ca^{++} uptake.

DISCUSSION

The results presented in this paper demonstrate that chronic ethanol inhibits ATP-dependent Ca^{++} uptake in a manner which is calmodulin-dependent (Table 2, Group 3 and Table 3, Group 3). Chronic ethanol treatment did not alter ATP-independent Ca^{++} binding to synaptosomal membranes a finding which differs from previous reports of chronic ethanol and changes in $^{45}Ca^{++}$ binding to membranes [26,31]. Inhibition of ATP-dependent Ca^{++} uptake was also seen with EGTA treatment of membranes with no effect on ATP-independent Ca^{++} uptake (Table 3, Group 1 vs. Table 2, Group 1). Addition of Calmodulin to ethanol treated membranes (Table 2, Group 4) reversed the effect of ethanol inhibition. Similarly, calmodulin addition to EGTA-treated ethanolic membranes reversed the ethanol effect (Table 3, Group 4). Combined EGTA treatment and ethanol consumption produced no further inhibition of ATP-dependent Ca^{++} uptake compared to EGTA alone (Table 3, Groups 1 and 3). This finding suggests that the pool of calmodulin necessary for ATP-dependent Ca^{++} uptake is affected similarly by EGTA and ETOH and supports the suggestion that ethanol acts to disrupt calmodulin·Ca^{++}·membrane activity. Earlier reports [34,35] have suggested that various particulate pools of calmodulin exist with varying dependencies on membrane Ca^{++} [36,37]. Based on the present findings ATP-independent Ca^{++} binding is unaffected by EGTA, ETOH or their combination and is probably not the pool of Ca^{++} which contributes to calmodulin activation of Ca^{++} uptake. It is not surprising that these findings differ from earlier reports by Michaelis and Myers [26] and Ross [32] since these reports did not control for free Ca^{++} with Ca^{++} buffers and probably measured a low affinity pool of Ca^{++} readily displaced by ethanol. Thus, the bound/free distribution of calmodulin important for ATP-dependent Ca^{++} uptake appears to be dependent on of both KCa^{++} and $K_{calmodulin}$ for this ATP-dependent reaction.

These findings are supported by recent studies which demonstrate that chronic drug treatment using agents which are known to alter endogenous pools of calcium from neuronal membranes [33] alter calmodulin distribution. Thus, O'Callaghan *et al.* [28], Clouet and Williams [4] and Ehrlich *et al.* [7,8] have demonstrated that chronic opiate and neuroleptic [14] treatment produced a change in the bound/free ratio of calmodulin distribution. Nehmad *et al.* [27] and Hoss *et al.* [18] have also demonstrated opiate alteration in calmodulin distribution.

In contrast, Luthin and Tabakoff [23] reported levels and subcellular distribution of calmodulin unchanged following 0–24 hr after withdrawal of mice from ethanol. Calmodulin-dependent changes in cAMP formation were also unchanged. These studies while focused on the adenylate cyclase-CM system do not compare to our studies since the time course for ethanol administration and biochemical system tested were different.

The functional significance of the present studies may lie in the apparent correlation of behavioral tolerance with decreased Ca^{++} buffering capacity. Inhibition of ATP-dependent Ca^{++} buffering capacity at the membrane level may act to increase more·Ca^{++}· available for intracellular metabolism. The rise in cytosolic Ca^{++} via this mechanism or via inhibition of Ca^{++}/Mg^{++} ATPase or Na^{+}/Ca^{++} exchange may influence changes in K^{+} conductance as previously reported [6]. Recent studies by Lynch *et al.* [25] suggest that sensitivity to Ca^{++} at the presynaptic terminal is increased during tolerance, as a possible adaptative mechanism. Increased Ca^{++} sensitivity may develop as a result of mobilization of intracellular Ca^{++}, not necessarily as a shift in releasable neurotransmitter pools [25], although a combination of these two mechanisms may be favored. Increased Ca^{++} availability may be achieved by limiting Ca^{++} buffering mechanisms giving rise to cytosolic Ca^{++} as is suggested by the present studies. Thus, increased Ca^{++} availability would be expected to increase the coupling event for a constant amount of transmitter available. Support for this hypothesis may be found from the studies of Lucchi *et al.* [22]. This study demonstrates differences in ^{3}H-nitrendipine binding which may be attributable to increased Ca^{++} sensitivity.

In summary, the development of pharmacological tolerance shown here occurs in parallel with decreases in calmodulin-dependent ATP stimulated Ca^{++} uptake. These findings suggest that tolerance may occur as a result of biochemical events in synaptosomal membranes which may contribute to increased intracellular Ca^{++} in nerve terminals.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs. Pat Hart for editorial assistance. This research was sponsored, in part, by a grant from the U.S. Brewers Association to David H. Ross.

REFERENCES

1. Blaustein, M. P., R. W. Ratzlaff, N. C. Kendrick and E. S. Schweitzer. Calcium buffering in presynaptic nerve terminals. Evidence for involvement of a nonmitochondrial ATP-dependent sequestration mechanism. *J Gen Physiol* **72:** 15–41, 1978.
2. Blaustein, M. P., R. W. Ratzlaff and E. S. Schweitzer. Calcium buffering in presynaptic nerve terminals. II. Kinetic properties of nonmitochondrial Ca^{++} sequestration mechanisms. *J Gen Physiol* **72:** 43–66, 1978.
3. Cheung, W. Y. Cyclic 3', 5'-neucleotide phosphodiesterase demonstration of an activator. *Biochem Biophys Res Commun* **38:** 533–538, 1970.
4. Clouet, D. H. and N. Williams. The rate of calmodulin in opioid-induced changes in the phosphorylation of rat striatal synaptic membrane proteins. *Neurochem Res* **7:** 1135–1148, 1982.
5. Cotman, C. W. and D. A. Matthews. Synaptic plasma membranes from rat brain synaptosomes. Isolation and partial characterization. *Biochim Biophys Acta* **249:** 389–394, 1971.
6. Durand, D. and P. L. Carlen. Decreased neuronal inhibition *in vitro* after long term administration of ethanol. *Science* **224:** 1359–1361, 1984.
7. Ehrlich, Y. H., D. A. Bounet, L. B. Davis and E. H. Brunngraber. Decreased phosphorylation of specific proteins in neostriatal membranes from rats after long-term narcotic exposure. *Life Sci* **23:** 137–146, 1978.
8. Ehrlich, Y. H., L. B. Davis, P. Keen and E. H. Brunngraber. Endorphin-regulated protein phosphorylation in brain membranes. *Life Sci* **26:** 1765–1772, 1980.
9. Garrett, K. M. and D. H. Ross. Effects of *in vivo* ethanol administration on Ca^{++}/Mg^{++}ATPase and ATP-dependent Ca^{++} uptake activity in synaptosomal membranes. *Neurochem Res* **8:** 1013–1027, 1983.
10. Gnegy, M. E., A. Lurchelli and E. Costa. Correlation between drug-induced supersensitivity of dopamine-dependent striatal mechanisms and the increase in striatal content of the Ca^{++}-regulated protein activator of cAMP phosphodiesterase. *Naunyn Schmiedebergs Arch Pharmacol* **201:** 121–127, 1977.
11. Gnegy, M., P. Uzunor and E. Costa. Participation of an endogenous Ca^{++} binding protein activator in the development of drug-induced super-sensitivity of striatal dopamine receptors. *J Pharmacol Exp Ther* **202:** 564–588, 1977.
12. Gnegy, M. E. Relationship of calmodulin and dopaminergic activity in the striatum. *Fed Proc* **41:** 2272–2277, 1982.
13. Goldstein, D. B. and N. Pal. Alcohol dependence produced in mice by inhalation of ethanol: Grading the withdrawal reaction. *Science* **172:** 288–290, 1971.
14. Hanbauer, I., J. Gimble and W. Lovenberg. Changes in soluble calmodulin following activation of dopamine receptors in rat striatal slices. *Neuropharmacology* **18:** 851–857, 1979.
15. Hanbauer, I., S. Pradham and H.-Y. T. Yang. Role of calmodulin in dopaminergic transmission. *Ann NY Acad Sci* **356:** 292–303, 1980.
16. Harris, R. A. Ethanol and pentobarbital inhibition of intrasynaptosomal sequestration of calcium. *Biochem Pharmacol* **30:** 3209–3215, 1981.
17. Harris, R. A. and D. Ferner. Ethanol and synaptosomal calcium binding. *Biochem Pharmacol* **31:** 1790–1792, 1982.
18. Hoss, W. and M. Fermaniak. Effects of opiates on synaptosomal caldmodulin and calcium uptake. *Neurochem Res* **8:** 219–229, 1983.
19. Jarrett, H. W. and J. T. Penniston. Purification of the Ca^{++}-stimulated ATPase activator from human erythrocytes. *J Biol Chem* **253:** 4676–4682, 1978.
20. Katz, S. and M. A. Remtulla. Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum. *Biochem Biophys Res Commun* **83:** 1373–1379, 1978.
21. Lau, Y. S. and M. E. Gnegy. The effects of lanthanum and trifluroperazine on ^{124}I-calmodulin binding to rat striatal particulates. *J Pharmacol Exp Ther* **215:** 28–34, 1980.
22. Lucchi, L., E. Govoni, R. Ballaini, G. Pasinetti and M. Trabucchi. Ethanol administration *in vivo* alters calcium ions control in rat striatum. *Brain Res* **332:** 376–379, 1985.
23. Luthin, G. and B. Tabakoff. Effects of ethanol on calmodulin levels in mouse striatum and cerebral cortex. *Alcohol Clin Exp Res* **8:** 68–72, 1984.
24. Luthra, M. G., K. S. Su and D. J. Hanahan. Purification of an activator of human erythrocyte membrane (Ca^{++}-Mg^{++}) ATPase. *Biochem Biophys Res Commun* **77:** 678–687, 1977.
25. Lynch, M. A., C. Pagonis, D. Samuel and J. Littleton. Alterations in Ca^{++}-dependent and Ca^{++}-independent release of catecholamines in preparations of rat brain produced by ethanol treatment *in vivo*. *Alcohol* **2:** 139–144, 1985.
26. Michaelis, E. K. and S. L. Myers. Calcium binding to brain synaptosomes. *Biochem Pharmacol* **28:** 2081–2087, 1979.
27. Nehmad, R., H. Nadler and R. Simantov. Effects of acute and chronic morphine treatment on calmodulin activity of rat brain. *Mol Pharmacol* **22:** 389–394, 1982.
28. O'Callaghan, J. P., N. Williams and D. H. Clouet. The effects of morphine on endogenous phosphorylation of synaptic plasma membrane proteins of rat striatum. *J Pharmacol Exp Ther* **208:** 96–105, 1979.
29. O'Callaghan, J. P., J. C. Juskewich and W. Lovenberg. The effects of morphine on calcium-related phosphorylation of synaptosomal cytosolic proteins from rat striatum. *J Pharmacol Exp Ther* **220:** 696–702, 1982.
30. Ritzmann, R. F. and B. Tabakoff. Body temperature in mice: A quantitative measure of alcohol tolerance and physical dependence. *J Pharmacol Exp Ther* **199:** 62–68, 1976.
31. Ross, D. H. Adaptive changes in Ca^{++} membrane interactions following chronic ethanol exposure. *Adv Exp Med Biol* **85A:** 459–465, 1977.
32. Ross, D. H. and H. L. Cardenas. Calmodulin stimulation of Ca^{++}-dependent ATP hydrolysis and ATP-dependent Ca^{++} transport in synaptic membranes. *J Neurochem* **41:** 161–171, 1983.
33. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol Rev* **24:** 583–655, 1972.
34. Sobue, K., S. Ichida, H. Yoshida, R. Yamazaki and S. Kakeuchi. Occurrence of a Ca^{++} and modulator protein activatable ATPase in the synaptic plasma membranes of brain. *FEBS Letts* **99:** 199–202, 1979.
35. Sorenson, R. G. and H. F. Mahler. Calcium-stimulated adenosine triphosphates in synaptic membranes. *J Neurochem* **37:** 1407–1418, 1981.
36. Teshima, Y. and S. Kakuichi. Membrane bound forms of Ca^{++}-dependent protein modulator, Ca^{++}-dependent and -independent binding of modulator protein to the particular fraction from brain. *J Cyclic Nucleotide Res* **4:** 219–231, 1978.
37. Vandermeers, A., P. Robberecht, M. C. Vandermeers-Piret, J. Rathe and J. Christophe. Specific binding of calcium-dependent regulator proteins to brain membranes from guinea pig. *Biochem Biophys Res Commun* **84:** 1076–1081, 1978.
38. Williams, N. and D. H. Clouet. The effect of acute opioid administration on the phosphorylation of rat striatal synaptic membrane proteins. *J Pharmacol Exp Ther* **220:** 278–286, 1982.