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EFFECTS OF ETHANOL AND BARBITURATES ON Ca^{2+}-ATPase ACTIVITY OF ERYTHROCYTE AND BRAIN MEMBRANES

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Abstract—Exposure to ethanol or pentobarbital *in vitro* stimulated the ATP-dependent efflux of calcium from human red blood cells (RBC) and the Ca^{2+}-ATPase activity of RBC and rat brain synaptic plasma membranes (SPM). These effects were obtained with concentrations of ethanol (50 mM) and pentobarbital (60 μM) associated with intoxication *in vivo*. The enhancement of SPM Ca^{2+}-ATPase by ethanol was due to an increase in the apparent affinity of the enzyme for calcium with no change in the maximum velocity. SPM Ca^{2+}-ATPase was also stimulated by an unsaturated fatty acid, *cis*-vaccenic acid methyl ester (*cis*-VAME). The membrane-disordering effects of ethanol, four barbiturates and *cis*-VAME were evaluated in SPM using the fluorescent probe molecule 1,6-diphenyl-1,3,5-hexatriene (DPH). All the compounds decreased the fluorescence polarization of DPH, and these decreases were proportional to the increase in Ca^{2+}-ATPase produced by these drugs. These findings suggest that the increase in Ca^{2+}-ATPase and calcium efflux produced by ethanol and pentobarbital results from the membrane-disordering effects of these drugs.

Calcium is a key regulator of cellular processes, particularly in the nervous system, where neurotransmitter release is regulated by intracellular levels of free calcium [1]. For calcium to act as an effective messenger, intracellular levels of free calcium must be maintained at low levels. A number of cells, including those of the nervous system, have an energy-dependent pump to aid in the extrusion of cytoplasmic calcium. Active transport of calcium is accomplished by a Ca^{2+}-ATPase located in the plasma membrane [2]. This process has been characterized most thoroughly in the human red blood cell (RBC). This cell is ideal for the measurement of calcium fluxes and ATPase activity because it does not contain mitochondria or other intracellular organelles to sequester calcium, it may be lysed and resealed with known amounts of calcium, and its plasma membrane is readily isolated [2]. There is evidence that the activity of Ca^{2+}-ATPase is altered by changes in the order of the membrane lipids surrounding it [3, 4] and may thereby be influenced by membrane perturbants. Intoxicant-anesthetic drugs such as ethanol and barbiturates are known to disorder biological membranes [5, 6] and to alter neuronal calcium transport [7]. Preliminary reports indicate that *in vitro* exposure to ethanol increases the Ca^{2+}-ATPase activity of RBC, and acute injection of ethanol enhances this activity in brain synaptic plasma membranes (SPM) [8, 9]. *In vitro* exposure of SPM to sedative barbiturates also increases SPM Ca^{2+}-ATPase [10], although *in vitro* exposure to ethanol has been reported to decrease this activity [11]. Thus, the effects of ethanol observed *in vitro* are the opposite of those observed *in vivo*. In all of these studies, the effect of ethanol was rather small (i.e. 10–20% change at 200 mM *in vitro* or 2 g/kg *in vivo*). Furthermore, each of these reports has involved a somewhat different assay for Ca^{2+}-ATPase. Because of the complex relationship between ATPase activity and the concentrations of calcium, magnesium and ATP [12, 13], it is difficult to compare results between these studies. In view of these considerations, we designed experiments to answer the following questions: (1) Does *in vitro* exposure to ethanol or pentobarbital alter the ATP-dependent efflux of calcium from RBC? (2) Does ethanol or pentobarbital alter the Ca^{2+}-ATPase activity of RBC or SPM when these tissues are assayed under the same conditions? (3) Are the effects of ethanol and barbiturates on Ca^{2+}-ATPase due to their membrane-disordering effect? The results presented here indicate that ethanol and pentobarbital increase ATP-dependent calcium efflux and Ca^{2+}-ATPase and suggest that these actions are due to membrane perturbation.

MATERIALS AND METHODS

Drugs. *R*(+) and *S*(−) isomers of pentobarbital were provided by the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD), 5-ethyl-5-(2-cyclohexylideneethyl)barbituric acid (CHEB) was provided by Dr. H. Downes, University of Oregon (Portland, OR), and racemic sodium pentobarbital and *cis*-vaccenic acid methyl ester (*cis*-VAME) were purchased from the Sigma Chemical Co. (St. Louis, MO). The purity of *cis*-

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VAME was 97–99%, as determined by gas chromatography.

Efflux of $^{45}Ca^{2+}$ from human RBC. Blood was obtained from the University of Missouri Hospital Blood Bank, was stored at 4° after being drawn, and was 2- to 10-days-old at the time of use. The procedure for incorporation of ATP and $^{45}Ca^{2+}$ into RBC was similar to that of Schatzmann [14]. RBC were washed three times with 140 mM NaCl (pH 7.4) and lysed by suspending in 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) containing 0.01 mM $CaCl_2$, 2 mM $MgCl_2$, $^{45}Ca^{2+}$ and 0 or 1.6 mM Tris-ATP (Sigma Chemical Co., Cat. No. A0520) at 4°. After 130 sec, the cells were resealed by restoration of isotonicity by addition of 3 M KCl at room temperature. Cells were quickly washed twice with incubation medium (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Tris, pH 7.4). Cells were resuspended in incubation medium and incubated at 33° with appropriate concentrations of ethanol or pentobarbital. After 5 or 10 min, RBC were pelleted by centrifugation for 2 min (Microfuge, Fisher Scientific, St. Louis, MO). Each sample was assayed in duplicate. The efflux of calcium was determined by measuring the amount of $^{45}Ca^{2+}$ in the supernatant fraction by liquid scintillation spectrometry. The ATP-dependent efflux was calculated as the difference in efflux between cells resealed with ATP and without ATP.

Preparation of brain SPM. Male Sprague–Dawley rats (Sasco, Inc., Indianapolis, IN) were killed by decapitation, and synaptic membranes (SPM1 + SPM2) were prepared from a whole brain homogenate by a modification [15] of the method of Cotman and Matthews [16]. Unless otherwise noted, SPM were not stored but were used immediately after preparation for the assay of ATPase.

Determination of ATPase. ATPase activity was measured by the colorimetric determination of phosphate hydrolyzed from ATP following the approach of Sorensen and Mahler [12]. RBC ghost membranes were prepared from freshly obtained blood by lysing washed RBC (described above) with 20 mM Tris, pH 8.1, for 10 min and washing with 50 mM imidazole, pH 7.4. To measure Ca^{2+}-ATPase, RBC ghosts or SPM were incubated with 1 ml of 50 mM imidazole (pH 7.4) containing 1 mM Tris-ATP, appropriate concentrations of ethanol or barbiturates, and 0.01 mM $CaCl_2$ or 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) at 37°. Incubation time was either 10 or 20 min (see figures). Samples were placed on ice, and the amount of inorganic phosphate produced was determined [17]. Ca^{2+}-ATPase activity was calculated as the difference in hydrolysis of ATP in the presence of $CaCl_2$ and in the presence of EGTA (no calcium). Mg^{2+}-ATPase was measured in the same way as Ca^{2+}-ATPase, except that 2 mM $MgCl_2$ was substituted for $CaCl_2$. To study the effect of *cis*-VAME on Ca^{2+}-ATPase, SPM (5 ml) were incubated with 0.3 mM *cis*-VAME for 30 min on ice [18]. *cis*-VAME was dissolved in ethanol and the final concentration of ethanol was 2%, v/v. A control group was incubated with ethanol (2%, v/v) alone. To remove the ethanol and unincorporated *cis*-VAME, SPM were pelleted and washed twice with 20 ml of incubation buffer. Membranes were suspended in incubation buffer, and Ca^{2+}-ATPase and fluorescence polarization were determined immediately. No residual ethanol was detected (gas chromatography) in the final membrane suspension.

Fluorescence polarization. Effects of ethanol, barbiturates and *cis*-VAME on membrane fluidity were evaluated by fluorescence polarization [6, 19]. The fluorescent probe molecule, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Fluka, Tridom Chemical Inc., Hauppauge, NY), was incorporated into SPM suspension (50 μg protein/ml) at a concentration of 0.5 μg/ml. The probe was dissolved in tetrahydrofuran (THF), and a volume of 0.5 μl THF/ml was added directly to the membrane suspension. The suspension was incubated in the dark at 35° and was vortexed frequently until maximal fluorescence was obtained (20–30 min). After incorporation of probe, the membrane suspensions were placed in quartz cuvettes and the temperature was maintained at 35°. Control levels of fluorescence were determined and an aliquot (1–100 μl) of a drug solution was then added to the cuvette and fluorescence was again determined. Fluorescence polarization (P) was determined with an HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, CA) with fixed polarization filters [6].

Other methods. Protein was determined by the phenol method [20]. Effects of drug treatments were evaluated statistically by Student's *t*-test for paired samples.

RESULTS

Effects of ethanol on efflux of $^{45}Ca^{2+}$ from RBC. Human RBC were lysed and resealed with $^{45}Ca^{2+}$ with or without ATP, allowing measurement of ATP-dependent and ATP-independent efflux of calcium from these cells. The ATP-dependent efflux was three times larger than the ATP-independent process. *In vitro* addition of ethanol stimulated the ATP-dependent efflux (Fig. 1). An enhancement of about 30% was produced by a concentration of 50 mM, and higher ethanol concentrations produced no additional increase. ATP-dependent efflux increased linearly between incubation periods of 1–10 min (not shown). The relative stimulation of efflux by ethanol was similar for 5- and 10-min incubation times (Fig. 1). ATP-independent efflux of calcium was not altered by ethanol (not shown).

Effects of ethanol and pentobarbital on Ca^{2+}-ATPase. The stimulation of ATP-dependent calcium efflux by ethanol raised the possibility that ethanol might produce this effect by increasing the activity of Ca^{2+}-ATPase. Before studying drug effects on Ca^{2+}-ATPase, it was necessary to determine appropriate storage conditions for RBC and brain membranes. Freezing for 20 hr completely abolished the Ca^{2+}-ATPase activity of both RBC membranes and SPM, while storage of SPM on ice for 27 hr reduced activity by about 25% (Fig. 2). With fresh preparations, the Ca^{2+}-ATPase activity of SPM was about fifteen times greater than that of RBC membranes. Because of the loss of enzymatic activity with storage,

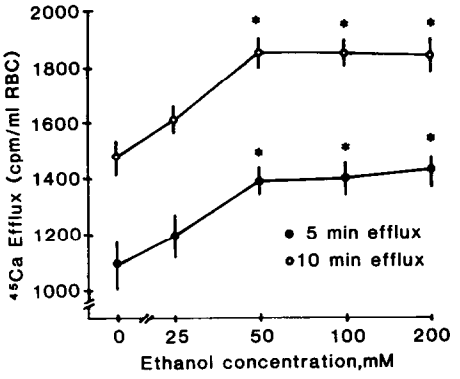

Fig. 1. Effects of ethanol on the ATP-dependent efflux of $^{45}Ca^{2+}$ from red blood cells. Ethanol was added *in vitro* at the concentration (mM) indicated on the abscissa. The ordinate represents the ATP-dependent $^{45}Ca^{2+}$ efflux. Closed circles represent a 5-min efflux period; open circles represent a 10-min efflux period. Each value is the mean ± S.E.M. of duplicate determinations of four membrane preparations. Asterisks indicate that ethanol concentrations greater than 25 mM significantly ($P < 0.01$) increased efflux.

all other experiments were performed immediately after preparation of membranes.

Ca^{2+}-ATPase activity of RBC membranes was increased by *in vitro* addition of ethanol (Fig. 3). The enhancement was similar to that observed for calcium efflux, with 50 and 100 mM concentrations of ethanol producing 20–30% increase in Ca^{2+}-ATPase and 200 mM producing no additional increase. (±)-Pentobarbital also stimulated the activity of the enzyme with 0.06 mM producing an effect similar to that of 50 mM ethanol (Fig. 3). The Ca^{2+}-ATPase activity of SPM was also increased by *in vitro* addition of ethanol (Fig. 4). A concentration

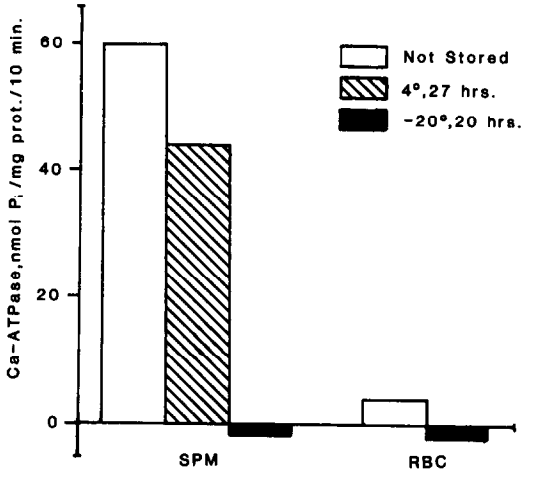

Fig. 2. Effects of storage conditions on the Ca^{2+}-ATPase of red blood cells (RBC) and synaptic plasma membranes (SPM). Open bars repesent Ca^{2+}-ATPase activity of membranes assayed immediately after preparation, solid bars represent the activity of membranes assayed after storage at −20° for 20 hr and the hatched bar represents the activity of SPM after storage at 4° for 27 hr. Each value is the mean of six determinations.

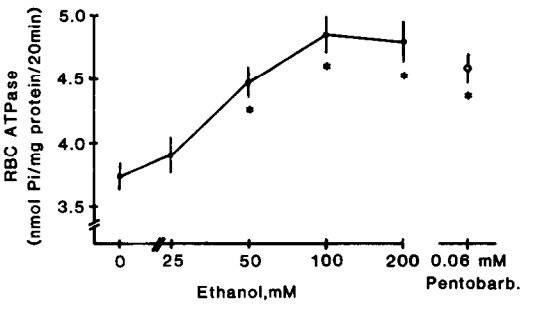

Fig. 3. Effects of ethanol and pentobarbital on the Ca^{2+}-ATPase activity of red blood cell (RBC) membranes. No drug (control), ethanol or pentobarbital was added *in vitro* at the concentration indicated on the abscissa. Ca^{2+}-ATPase activity is indicated on the ordinate. Values represent mean ± S.E.M., N = 4. Asterisks denote a significant drug effect, $P < 0.01$.

of 100 mM increased activity by about 35%; thus, the magnitude of the effect was similar to that observed with RBC membranes.

All of the results presented above were obtained using a calcium concentration of 0.01 mM. The effects of ethanol on the Ca^{2+}-ATPase of SPM were also tested in the presence of higher concentrations of calcium. Increases in calcium resulted in higher Ca^{2+}-ATPase, but the effect of ethanol was eliminated as calcium concentrations approached 0.1 mM (Table 1).

Comparison of drug effects on SPM Ca^{2+}*-ATPase and membrane order.* Stimulation of Ca^{2+}-ATPase was also observed with the resolved isomers of pentobarbital, with the barbiturate derivative CHEB, and with the unsaturated fatty acid *cis*-VAME (Fig. 5). The ability of such chemically diverse compounds to stimulate Ca^{2+}-ATPase suggested that they might produce their effects by perturbing membrane lipids. This possibility was investigated by measuring the polarization of DPH fluorescence. This parameter is a sensitive measure of the ordering of membrane

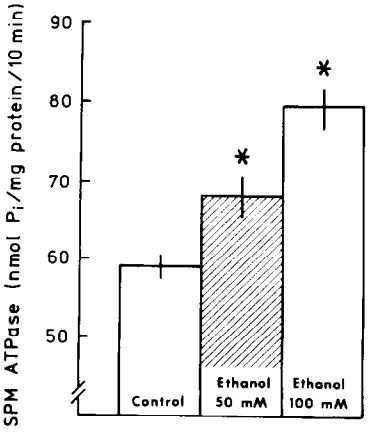

Fig. 4. Effects of ethanol on the Ca^{2+}-ATPase activity of brain synaptic plasma membranes (SPM). Ethanol was added *in vitro* at a concentration of 50 or 100 mM. The ordinate indicates the activity of Ca^{2+}-ATPase. Bars represent mean ± S.E.M., N = 4. Asterisks denote a significant effect of ethanol, $P < 0.01$.

Table 1. Effects of ethanol on Ca^{2+}-ATPase activity of SPM as a function of calcium concentration

Ca^{2+} concn (mM)	Ca^{2+}-ATPase activity*	
	Control	Ethanol (100 mM)
0.01	58 ± 4	80 ± 5†
0.02	94 ± 4	126 ± 7†
0.08	192 ± 9	178 ± 8
0.60	369 ± 24	310 ± 18

* Values represent mean ± S.E.M., N = 4, and are expressed as nmoles P_i per mg protein per 10 min.

† Significantly different from control, $P < 0.01$ (t-test for paired observations).

lipids [21]. All of the drug treatments decreased the polarization of fluorescence (Fig. 5), indicating a disordering of the synaptic membrane. The potencies of the drugs varied greatly but, at the concentrations tested, the relative magnitude of the decrease in polarization was similar to the increase in Ca^{2+}-ATPase activity. Linear regression analysis of the data in Fig. 5 indicated a correlation ($r = 0.95$) between the change in fluorescence polarization and the percent increase in Ca^{2+}-ATPase activity.

Effects of ethanol and pentobarbital on Mg^{2+}-*ATPase.* ATPase activity of SPM and RBC membranes was also stimulated by magnesium and, as was demonstrated for Ca^{2+}-ATPase, Mg^{2+}-ATPase was much higher for SPM than for RBC (Fig. 6). Ethanol and pentobarbital failed to alter the activity of Mg^{2+}-ATPase.

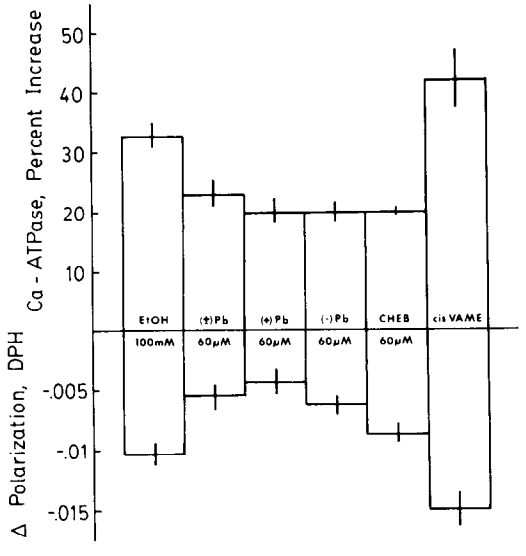

Fig. 5. Comparison of the effects of drugs on Ca^{2+}-ATPase and synaptic membrane fluidity. The upper half represents the drug-induced increases in SPM Ca^{2+}-ATPase as a percentage of the control (no drug) activity. The lower panel represents the fluorescence polarization of DPH incorporated into SPM. The bars represent effects of the following drugs (left to right): ethanol (100 mM), (±)pentobarbital (60 μM), $R(+)$pentobarbital (60 μM), $S(-)$pentobarbital (60 μM), CHEB (60 μM), and *cis*-VAME (0.3 mM). Values are the mean ± S.E.M., N = 6. All drugs produced significant ($P < 0.01$) increases in Ca^{2+}-ATPase and decreases in fluorescence polarization.

DISCUSSION

The evidence presented here, derived both from measurement of calcium transport and enzyme activity, demonstrates that ethanol and pentobarbital increased the Ca^{2+}-ATPase of the plasma membranes of erythrocytes and nerve endings. Plasma membranes of cells from liver and muscle also contain a Ca^{2+}-ATPase [22, 23], and our results raise the possibility that ethanol alters calcium transport in other tissues. The stimulation of Ca^{2+}-ATPase was observed with pharmacologically relevant concentrations of the drugs, as the concentrations of ethanol (50 mM) and pentobarbital (60 μM) which produced significant effects are associated with moderate to severe intoxications *in vivo* [24, 25]. The increase in Ca^{2+}-ATPase activity amounted to 20–40% with a calcium concentration of 10 μM. The calcium dependence of the effect indicated, however, that the enhancement is greater at lower concentrations of calcium. Because intracellular concentrations of free calcium are between 1 and 0.01 μM *in vivo* [26], it is likely that the effects of the drugs are even greater under these conditions.

Several lines of evidence indicate that the stimulation of Ca^{2+}-ATPase by ethanol and barbiturates was due to their membrane-disordering action. First, three chemically diverse membrane perturbants, ethanol, pentobarbital and *cis*-VAME, all increased enzyme activity. Second, the increase in Ca^{2+}-ATPase produced by six different compounds was proportional to their abilities to disorder the synaptic membrane. Third, the optical isomers of pentobarbital were equally effective in stimulating Ca^{2+}-ATPase (and in disordering the membrane) despite their stereoselective interaction with some membrane proteins [27]. Fourth, other studies indicate that biochemical manipulations which increase membrane fluidity also enhance Ca^{2+}-ATPase activity [4, 28]. An important aspect of the present study is that it suggests that the membrane perturbation produced by pharmacologically relevant concentrations of ethanol and pentobarbital was sufficient to alter membrane function. Demonstration of functional consequences of the membrane perturbation produced by low concentrations of these drugs is a crucial component of the "disordered lipid" hypothesis of anesthetic action which has seldom been addressed experimentally [29].

The observed stimulation of SPM Ca^{2+}-ATPase suggests that ethanol and barbiturates enhance the efflux of calcium from nerve cells. Studies with isolated synaptosomes indicate that pentobarbital does indeed stimulate $^{45}Ca^{2+}$ efflux, but this effect was not observed with ethanol or CHEB [30, 31]. Interpretation of these results is difficult due to the complexity of calcium transport in synaptosomes. For example, the effects of ethanol on calcium efflux were measured under conditions where both sodium–calcium exchange and Ca^{2+}-ATPase contribute to efflux. There is now evidence that ethanol inhibits sodium–calcium exchange [32], and this effect may have obscured an increase in ATP-dependent efflux. In addition, the efflux studies require loading of the synaptosomes with calcium. (In contrast to RBC, intrasynaptosomal calcium cannot be reliably

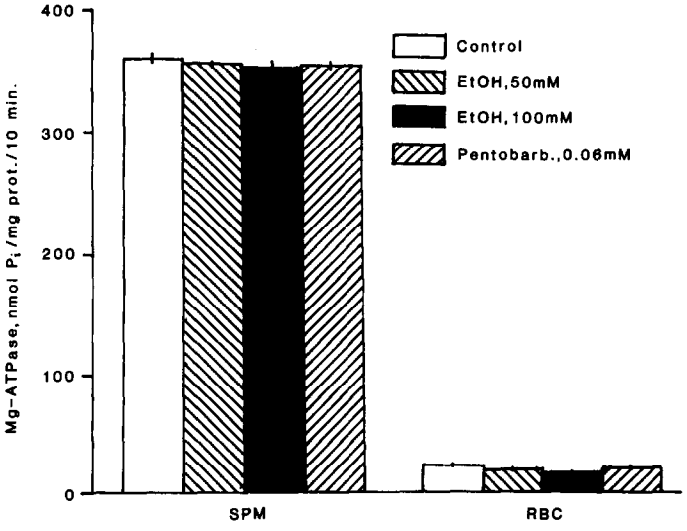

Fig. 6. Effects of ethanol and pentobarbital on Mg^{2+}-ATPase activity of SPM and RBC membranes. For each membrane, the bars represent the following treatments (left to right): no drug (control), ethanol (50 mM), ethanol (100 mM), and pentobarbital (0.06 mM). The ordinate represents the activity of Mg^{2+}-ATPase. Values are mean ± S.E.M., N = 4.

adjusted by resealing with known amounts of calcium.) Because the effects of drugs on Ca^{2+}-ATPase were diminished as the calcium concentration was increased, preloading with excessive calcium may reduce the effects of the drugs on ATP-dependent efflux.

In summary, the present results, together with those from other studies [7], indicate that the Ca^{2+}-ATPase of the neuronal plasma membrane is one of several sites responsible for the actions of alcohols and barbiturates on neuronal calcium homeostasis.

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REFERENCES

1. R. P. Rubin, *Calcium and Cellular Secretion*. Plenum Press, New York (1982).
2. H. J. Schatzmann, in *Membrane Transport of Calcium* (Ed. E. Carafoli), p. 41. Academic Press, New York (1982).
3. H. Sandermann, Jr. *Biochim. biophys. Acta* **515**, 209 (1978).
4. B. M. Moore, B. R. Lentz, M. Hoechli and G. Meissner, *Biochemistry* **20**, 6810 (1981).
5. J. H. Chin and D. B. Goldstein, *Molec. Pharmac.* **13**, 435 (1977).
6. R. A. Harris and F. Schroeder, *J. Pharmac. exp. Ther.* **223**, 424 (1982).
7. R. A. Harris, in *New Perspectives on Calcium Antagonists* (Ed. G. B. Weiss), p. 223. American Physiological Society, Bethesda, MD (1981).
8. B. J. Logan and R. Laverty, *Proc. Univ. Otago med. Sch.* **59**, 49 (1980).
9. D. H. Ross, T. L. Mutchler and M. M. Grady, *Alcoholism: Clin. expl. Res.* **3**, 64 (1979).
10. M. Willow and G. A. R. Johnston, *Neurosci. Lett.* **14**, 361 (1979).
11. A. Y. Sun and R. N. Seaman, *Neurochem. Res.* **5**, 537 (1980).
12. R. G. Sorensen and H. R. Mahler, *J. Neurochem.* **37**, 1407 (1981).
13. H. A. Pershadsingh and J. M. McDonald, *J. biol. Chem.* **255**, 4087 (1980).
14. H. J. Schatzmann, *J. Physiol. Lond.* **235**, 551 (1973).
15. W. F. Hood and R. A. Harris, *Biochem. Pharmac.* **28**, 3075 (1979).
16. C. W. Cotman and D. A. Matthews, *Biochim. biophys. Acta* **249**, 380 (1971).
17. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
18. J. Orly and M. Schramm, *Proc. natn. Acad. Sci. U.S.A.* **72**, 3433 (1975).
19. R. A. Harris and F. Schroeder, *Molec. Pharmac.* **20**, 128 (1981).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. M. Shinitzky and Y. Barenholz, *Biochim. biophys. Acta* **515**, 367 (1978).
22. A. Cittadini and G. D. V. van Rossum, *J. Physiol. Lond.* **281**, 29 (1978).
23. R. Casteels and C. van Breemen, *Pflügers Archs* **359**, 197 (1975).
24. I. K. Ho and R. A. Harris, *A. Rev. Pharmac.* **21**, 83 (1981).
25. K. V. Tullis, W. Q. Sargent, J. R. Simpson and J. D. Beard, *Life Sci.* **20**, 875 (1977).
26. C. F. McGraw, D. A. Nachshen and M. P. Blaustein, in *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. II, p. 81, Academic Press, New York (1982).
27. M. K. Ticku, *Brain Res.* **211**, 127 (1981).
28. G. Schmalzing and P. Kutschera, *J. membr. Biol.* **69**, 65 (1982).
29. R. A. Harris and R. J. Hitzemann, in *Currents in Alcoholism*, (Ed. M. Galanter), Vol. VIII, p. 379. Grune & Stratton, New York (1981).
30. R. A. Harris and W. F. Hood, *J. Pharmac. exp. Ther.* **213**, 562 (1980).
31. R. A. Harris and J. A. Stokes, *Brain Res.* **242**, 157 (1982).
32. M. L. Michaelis and E. K. Michaelis, *Alcoholism: Clin. expl. Res.* **6**, 306 (1982).