Pang, D. T., Lewis, S. D., Sharma, B. R., & Shafer, J. A. (1984) *Arch. Biochem. Biophys. 234*, 629–638.
Pilch, P. F. (1985) in *Insulin Receptors* (Piro, R. D., & Lauro, R., Eds.) pp 33–43, Acta Medica, Rome.
Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3381.
Pilch, P. F., & Czech, M. P. (1980) *J. Biol. Chem. 255*, 1722–1731.
Pilch, P. F., Axelrod, J. D., & Czech, M. P. (1981) in *Current Views on Insulin Receptors* (Andeani, D., et al., Eds.) pp 255–260, Academic, London.
Rosen, O. M., Herrera, R., Olowe, Y., Petrucelli, L. M., & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3237–3240.
Roth, R. A., & Cassell, D. J. (1983) *Science (Washington, D.C.) 219*, 299–301.
Roth, R. A., Mesirow, M. L., & Cassell, D. J. (1983) *J. Biol. Chem. 258*, 14456–14460.
Salzman, A., Wan, C. F., & Rubin, C. F. (1984) *Biochemistry 23*, 6555–6565.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Shia, M. A., & Pilch, P. F. (1983) *Biochemistry 22*, 717–721.
Shia, M. A., Rubin, J. B., & Pilch, P. F. (1983) *J. Biol. Chem. 258*, 14450–14455.
Sweet, L. J., Morrison, B. D., & Pessin, J. E. (1987) *J. Biol. Chem. 262*, 6939–6942.
Switzer, R. C., Merril, C. R., & Schifrin, S. (1979) *Anal. Biochem. 98*, 231–237.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Lia, Y.-L., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.
Van Obberghen, E., Kasuga, M., LeCam, A., Hedo, J. A., Itin, A., & Harrison, L. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1052–1056.
Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 945–949.
Welinder, B. S., Linde, S., Hansen, B., & Sonne, O. (1984) *J. Chromatogr. 298*, 41–57.
Yip, C. C., & Moule, M. L. (1983) *Diabetologia 32*, 760–767.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1978) *J. Biol. Chem. 253*, 1743–1745.

Effects of Chronic Exposure to Ethanol on the Physical and Functional Properties of the Plasma Membrane of S49 Lymphoma Cells†

Donald C. Bode‡ and Perry B. Molinoff*

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

Received September 23, 1987; Revised Manuscript Received March 15, 1988

ABSTRACT: The effects of chronic exposure to ethanol on the physical and functional properties of the plasma membrane were examined with cultured S49 lymphoma cells. The β-adrenergic receptor-coupled adenylate cyclase system was used as a probe of the functional properties of the plasma membrane. Steady-state fluorescence anisotropy of diphenylhexatriene and the lipid composition of the plasma membrane were used as probes of the physical properties of the membrane. Cells were grown under conditions such that the concentration of ethanol in the growth medium remained stable and oxidation of ethanol to acetaldehyde was not detected. Chronic exposure of S49 cells to 50 mM ethanol or growth of cells at elevated temperature resulted in a decrease in adenylate cyclase activity. There were no changes in the density of receptors or in the affinity of β-adrenergic receptors for agonists or antagonists following chronic exposure to ethanol. The fluorescence anisotropy of diphenylhexatriene was lower in plasma membranes prepared from cells that had been treated with 50 mM ethanol than in membranes prepared from control cells. However, this change was not associated with changes in the fatty acid composition or the cholesterol to phospholipid ratio of the plasma membrane. There was a small but statistically significant decrease in the amount of phosphatidylserine and an increase in the amount of phosphatidylethanolamine. These changes cannot account for the decrease in anisotropy. In contrast to the effect of ethanol, a decrease in adenylate cyclase activity following growth of S49 cells at 40 °C was not associated with a change in anisotropy.

The effects of ethanol on biological systems can be studied at several different levels. Studies with whole animals can be influenced by effects of ethanol on the physiological state of the animal as well as by metabolites of ethanol produced in the organ being studied or elsewhere in the body. For example, it has been shown that the toxic effects of acetaldehyde, the primary metabolite of ethanol, are evident at much lower concentrations than are required to see the effects of ethanol (Koerker et al., 1976). Thus, the effects of ethanol in whole animals may be partially or entirely due to acetaldehyde rather than to ethanol. Furthermore, in the whole animal, it may be difficult to determine the concentration of ethanol to which a particular tissue is exposed, and fluctuations in the concentration of ethanol occur, which can further complicate interpretation of results. The use of isolated organs and organ

† Supported by USPHS Grant AA 06215.
* Author to whom correspondence should be addressed.
‡ Present address: Department of Pharmacology, M-036, School of Medicine, University of California, San Diego, La Jolla, CA 92093.

0006-2960/88/0427-5700$01.50/0

cultures minimizes many of these problems, but, as in studies of whole animals, complexities arise from the presence of multiple cell types. Studies of subcellular fractions are easier to interpret but suffer from the fact that the integrity of the cell has been destroyed.

The use of clonal cultured cell lines circumvents many of these problems and provides a simple, homogeneous system in which the effects of ethanol on intact, living cells can be studied. The use of cultured cells also permits maintenance of a constant, known concentration of ethanol. Further, the growth medium can be assayed for acetaldehyde, which makes it possible to assess the metabolism of ethanol and the possible effects of acetaldehyde.

Results of a number of studies have established the utility of using cultured cells to investigate the acute and chronic effects of ethanol. Studies of the effects of ethanol have employed a variety of types of cultured cells, both primary cultures and established cell lines, from a number of different tissues. In most cases, cell viability was not affected adversely by ethanol concentrations comparable to those seen in vivo (less than 100 mM) [see Syapin and Noble (1979)]. In the one case where viability was decreased by a low concentration of ethanol (Walker et al., 1974), the decrease was probably due to production of acetaldehyde, since this effect was seen in a liver cell line (which was capable of metabolizing ethanol) but not in a line of fibroblasts.

Hill and Bangham (1975) proposed that dependence on central nervous system depressant, general anesthetic drugs (including ethanol) arises due to an alteration in the physical state of cell membranes that counteracts the direct, membrane-perturbing effect of the drugs. Dependence would occur when a drug is withdrawn abruptly, because the membranes would suddenly be in a state opposite to that which was produced by the drug.

Changes in membrane fluidity (Lyon & Goldstein, 1983; Polokoff et al., 1985) and in the sensitivity of membrane fluidity to ethanol in vitro (Chin & Goldstein, 1977; Harris et al., 1984a) following chronic ethanol treatment have been reported. According to the Hill and Bangham hypothesis, in the absence of ethanol in vitro, membranes from ethanol-treated animals should be less fluid than membranes from control animals, which would account for dependence. Furthermore, membranes from ethanol-treated animals should be more resistant to the disordering effect of ethanol in vitro than membranes from control animals, which could explain tolerance.

There are numerous reports of alterations in membrane lipid composition following exposure to ethanol. This has been seen not only in bacteria (Ingram, 1976) and the unicellular organism *Tetrahymena* (Nandini-Kishore et al., 1979) but also in higher animals. Changes in membrane lipid composition following chronic treatment with ethanol have also been reported in whole animals (Littleton & John, 1977; Chin et al., 1978), but the changes that have been reported are complex and variable and may depend on factors other than the presence or absence of ethanol, such as the species (or even the strain), the tissue, or the mode of administration of ethanol.

Several investigators have reported changes in the lipid composition of cultured mammalian cells following chronic exposure to ethanol, but they are often inconsistent with an adaptive response to the membrane-fluidizing effect of ethanol (Ingram et al., 1978; Keegan et al., 1983; Morrison et al., 1984). In Reuber H35 hepatoma cells, membrane fluidity increased following chronic exposure to ethanol, which may have been due to a decrease in the sphingomyelin content of the plasma membranes (Polokoff et al., 1985). The increase in fluidity, an inappropriate adaptive response to the acute fluidizing effect of ethanol, was prevented by an inhibitor of alcohol dehydrogenase, suggesting that the change was due to the production of acetaldehyde.

The activities of membrane-bound enzymes such as (Na^+,K^+)-ATPase and hormone-stimulated adenylate cyclase have been used to monitor changes in the functional properties of the plasma membrane following chronic exposure to ethanol. Thus, (Na^+,K^+)-ATPase in mouse brain synaptosomal membranes is inhibited by ethanol in vitro, and the enzyme from ethanol-tolerant animals is resistant to inhibition by ethanol (Levental & Tabakoff, 1980).

An involvement of dopamine receptors, some of which are linked to stimulation of adenylate cyclase activity, in tolerance to ethanol in mice has been suggested (Hoffman & Tabakoff, 1977). However, the decrease in dopamine-stimulated adenylate cyclase activity in striata from ethanol-tolerant mice reported by Hoffman and Tabakoff (1977) was not reproduced by Rabin et al. (1980). The acute and chronic effects of ethanol on hormone receptors and hormone-stimulated adenylate cyclase activity have also been studied using cultured mammalian cells (Stenstrom & Richelson, 1982; Charness et al., 1983; Gordon et al., 1986).

The goal of the present studies was to determine whether tolerance to or dependence on ethanol could be demonstrated at the biochemical level in cultured S49 lymphoma cells in terms of the effects of ethanol on the physical and functional properties of the plasma membrane. Such a comprehensive approach should be helpful in determining the relationship between changes in the physical and functional properties of the membrane that occur following chronic exposure to ethanol and the relevance of such changes to the development of tolerance to and dependence on ethanol. The β-adrenergic receptor-coupled adenylate cyclase system was used as a probe of the functional properties of the plasma membrane. The physical properties characterized included the membrane fluidity as measured by the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] and the lipid composition of the plasma membrane.

Materials and Methods

S49 lymphoma cells were grown in suspension culture in 75 cm^2 tissue culture flasks or spinner flasks (Bellco, Vineland, NJ). The growth medium consisted of Dulbecco's modification of Eagle's medium supplemented with 10% heat-inactivated horse serum in an atmosphere of 10% CO_2/90% air at 37 °C. Cell density was maintained between 1.5×10^5 and 2.0×10^6 cells/mL by dilution with fresh medium (with or without ethanol) every second day. Redistilled ethanol (95%) was added to the medium to achieve the desired final concentration prior to addition of cells.

The concentration of ethanol in tissue culture growth medium was determined by measuring the enzymatic reduction of NAD. A solution containing 500 mM glycine (pH 9.0), 56 mM semicarbazide hydrochloride, 50 units/mL alcohol dehydrogenase, and 0.6 mM NAD was added to 20 μL of medium containing up to 30 mM ethanol. The tubes were covered tightly and incubated at room temperature for 30 min.

[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic phosphate; DPH, 1,6-diphenyl-1,3,5-hexatriene; NAD, nicotinamide adenine dinucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; [^{125}I]IPIN, [^{125}I]iodopindolol.

Absorbance was measured at 340 nm.

The amount of acetaldehyde in tissue culture medium taken from cells that had been grown in the presence of ethanol was determined by head-space gas chromatography on a 6 ft long × 2 mm i.d. glass column containing 15% poly(ethylene glycol) on 80/100 mesh Gas Chrom Q, as described by Eriksson et al. (1977).

The rate of oxidation of ethanol was determined by measuring the conversion of [^{14}C]ethanol to $^{14}CO_2$. Wild-type S49 cells (2.0×10^6 cells) were incubated in 1.0 mL of growth medium containing 10 mM HEPES (pH 7.5), 50 mM ethanol, and 0.4 μCi of [^{14}C]ethanol at 37 °C for up to 120 min. The reactions were terminated at various times by addition of 0.1 mL of 1.0 N HCl from a syringe inserted through the stopper. After incubation overnight at 4 °C, the amount of radioactivity trapped by hyamine hydroxide was determined by liquid scintillation spectroscopy.

For assays of adenylate cyclase activity or for assays of β-adrenergic receptors, an unwashed membrane pellet was prepared. Cells were harvested by centrifugation at 600g for 10 min at room temperature, rinsed once by suspension in phosphate-buffered saline (138 mM NaCl, 4 mM KCl, 5 mM Na_2HPO_4, 2 mM KH_2PO_4, and 11 mM glucose) and centrifugation at 600g for 10 min at room temperature, and homogenized with a Brinkmann Polytron in cold buffer containing 2 mM HEPES (pH 7.5), 2 mM $MgCl_2$, and 1 mM EDTA (pH 7.5). Homogenates were centrifuged at 39000g for 25 min, and the pellets were resuspended in 2 mM HEPES (pH 7.5).

For measurement of steady-state fluorescence anisotropy or for analysis of membrane lipids, plasma membranes were purified by sucrose density gradient centrifugation (Ross et al., 1977).

Adenylate cyclase activity was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP by the method of Salomon et al. (1974), as modified by Minneman et al. (1979). Each assay contained, in a total volume of 200 μL, 50 mM HEPES (pH 7.5), 5 mM cAMP, 5 mM $MgCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.75 mM 3-isobutyl-1-methylxanthine, 0.25 mM ATP, 0.1 mg/mL creatine phosphokinase, 10 mM phosphocreatine, approximately 1 μCi of [α-^{32}P]ATP, various drugs, and membranes. The concentration of GTP, when present, was 50 μM. [^{3}H]cAMP (approximately 40 000 cpm) was used as an internal standard to determine the recovery of [^{32}P]cAMP. The recovery of [^{3}H]cAMP was generally at least 80%.

β-Adrenergic receptors were assayed by incubating membranes with [^{125}I]IPIN in a total volume of 250 μL of buffer containing 12 mM HEPES (pH 7.5), 0.54% NaCl, 1.0 mM $MgCl_2$, 4 μg/mL bovine serum albumin, and 0.5 mM ascorbic acid for 20 min at 37 °C. Reactions were terminated by dilution with 10 mL of wash buffer [10 mM Tris (pH 7.5) and 0.9% NaCl] at room temperature, followed by filtration through glass fiber filters (Schleicher & Schuell no. 30). Filters were washed with an additional 10 mL of wash buffer. Specific binding, defined as the amount of [^{125}I]IPIN bound in the absence of a competing ligand minus the amount bound in the presence of 50 μM (−)-isoproterenol plus 100 μM GTP, was generally greater than 90% of the total amount of [^{125}I]-IPIN bound. The total amount of [^{125}I]IPIN bound to the tissue was always less than 10% of the total amount of radioligand added to the assay. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

The steady-state fluorescence anisotropy of DPH was measured with an SLM Model 8000 spectrofluorometer (SLM Instruments, Urbana–Champaign, IL) with a temperature-controlled sample cavity. Plasma membranes [0.1 mg/mL protein in 20 mM HEPES (pH 7.5) and 0.9% NaCl] were incubated with 0.5 μM DPH at 37 °C for 15 min with continuous stirring. The concentrations of membrane protein and DPH were shown to be low enough to avoid depolarization due to light scattering and probe–probe interactions, respectively (data not shown). Probe molecules were excited with vertically polarized light at a wavelength of 360 nm. The fluorescence intensity at a wavelength of 429 nm was measured at an angle of 90° to the excitation beam. The intensity of the components of the fluorescence that were parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. The fluorescence anisotropy (r), which is inversely related to membrane fluidity, was calculated by using the equation:

$$r = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$$

Correction was made for differences in the efficiency of transmitting vertically and horizontally polarized light by the emission monochromator by determining the polarization ratio ($I_\parallel/I_\perp$) using horizontally rather than vertically polarized excitation light. The anisotropy of each 2.0-mL sample was first determined in the absence of drugs after equilibrium at the appropriate temperature for 5 min. Small volumes of redistilled 95% ethanol were added to achieve the desired final concentration, the sample was mixed, and the anisotropy was determined. In this way, the effect of the entire range of drug concentrations on the anisotropy was determined for each sample.

Lipids were extracted from purified plasma membranes using chloroform and methanol (Bligh & Dyer, 1959) in the presence of the antioxidant butylated hydroxytoluene. The amount of cholesterol in crude lipid extracts from purified plasma membranes was determined by gas chromatography on a 6 ft long × 2 mm i.d. glass column containing 3% OV-17 on 100/120 mesh Gas Chrom Q II run at 250 °C, with nitrogen as the carrier gas at a flow rate of 40 mL/min. Coprostanol was used as an internal standard (Sinensky, 1980). The amount of phosphorus in crude lipid extracts from purified plasma membranes was determined by the method of Bartlett (1959), with dimyristoylphosphatidylcholine as the standard. The phospholipids were separated by thin-layer chromatography in one dimension on high-performance silica gel plates with a preadsorbent zone (EM Science, Gibbstown, NJ), using a mobile phase containing denatured ethanol (Baker Proprietary Solvent), ammonium hydroxide, and chloroform in the proportions 50:10:6, respectively (Dugan, 1985). The plates were developed twice in the same dimension with the same mobile phase. This protocol allowed separation of cholesterol, phosphatidylethanolamine, cardiolipin, phosphatidylserine, phosphatidylcholine + phosphatidylinositol, sphingomyelin, and phosphatidic acid. In most experiments, phosphatidylcholine and phosphatidylinositol were not separated. For quantitation of individual phospholipids, bands corresponding to the location of phospholipid standards were scraped off the plates, and the phospholipids were determined by the method of Bartlett (1959). The fatty acyl chains of the phospholipids were converted to fatty acid methyl esters using 14% BF_3 in methanol (Morrison & Smith, 1964). Fatty acid methyl esters were separated by gas chromatography on a 10 ft long × 2 mm i.d. glass column containing 10% Silar 10C on 100/120 mesh Gas Chrom Q II, with nitrogen as the

carrier gas at a flow rate of 40 mL/min. The temperature was programmed to increase from an initial temperature of 190 °C for 4 min to 240 °C at 4 °C/min. Pentadecanoic acid methyl ester was used as the internal standard. The fatty acid methyl esters were quantitated by comparing their peak areas with that of the internal standard. Peaks were identified on the basis of comparison of their retention times with those of authentic standards. Correction was made for differences in the sensitivity of the detector to different fatty acid methyl esters by calculating the relative peak areas that were obtained with a mixture containing equal amounts, by weight, of all of the standards.

[α-^{32}P]ATP (>600 Ci/mmol) and [^{3}H]cAMP (approximately 30 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Carrier-free Na^{125}I (2200 Ci/mmol) was obtained from either New England Nuclear or Amersham (Arlington Heights, IL). [^{125}I]IPIN was synthesized from (−)-pindolol (a gift from Dr. Günter Engel of Sandoz Pharmaceuticals, Basel, Switzerland) and Na^{125}I by the chloramine-T method described by Wolfe and Harden (1981). Sigma (St. Louis, MO) was the source of isoproterenol, ATP, cAMP, GTP, cholesterol, and individual phospholipid and fatty acid methyl ester standards. Acetaldehyde was obtained from Aldrich (Milwaukee, WI). DPH was obtained from Aldrich and recrystallized from ethanol. Propranolol was a gift of Ayerst Laboratories (New York, NY). Fatty acid methyl ester standards and gas chromatography columns were purchased from Alltech Associates Inc. (Deerfield, IL). Coprostanol was obtained from Steraloids (Wilton, NH). Organic solvents were obtained from VWR or Fisher (King of Prussia, PA). S49 lymphoma cells were obtained from the Cell Culture Facility of the University of California, San Francisco. Dulbecco's modification of Eagle's medium was purchased from Flow Laboratories (McLean, VA) or GIBCO (Grand Island, NY). Heat-inactivated horse serum was obtained from HyClone Labs (Logan, UT).

Results

Conditions were established that were suitable for the use of cultured S49 lymphoma cells as a model system to study the effects of chronic exposure to ethanol on the physical and functional properties of the cell membrane. The system satisfied the following criteria: (1) the concentration of ethanol in the growth medium did not vary for at least 99 h, either in the presence or in the absence of cells; (2) ethanol caused a dose-dependent inhibition of the rate of growth of S49 cells, but adenylate cyclase activity was independent of variations in cell density; (3) there was no decrease in cell viability, as measured by Trypan blue exclusion, when cells were grown in the presence of up to 100 mM ethanol.

Experiments were carried out to determine whether S49 cells metabolize ethanol to acetaldehyde. Cells were incubated with [^{14}C]ethanol in a sealed vial, and the production of $^{14}CO_2$ was measured. There was no difference in the amount of $^{14}CO_2$ produced in the presence or in the absence of S49 cells (data not shown). The head-space gas over samples of growth medium was analyzed by gas chromatography to determine the concentration of acetaldehyde in the medium. When S49 lymphoma cells were grown in tightly capped flasks in the presence of 50 mM ethanol for up to 12 days, acetaldehyde was not detectable in the medium (data not shown). When cells were incubated in tightly closed flasks in medium that initially contained 45 μM acetaldehyde, no acetaldehyde remained after 2 days. In the absence of cells under the same conditions, the concentration of acetaldehyde after 2 days was 86% of the initial concentration (data not shown).

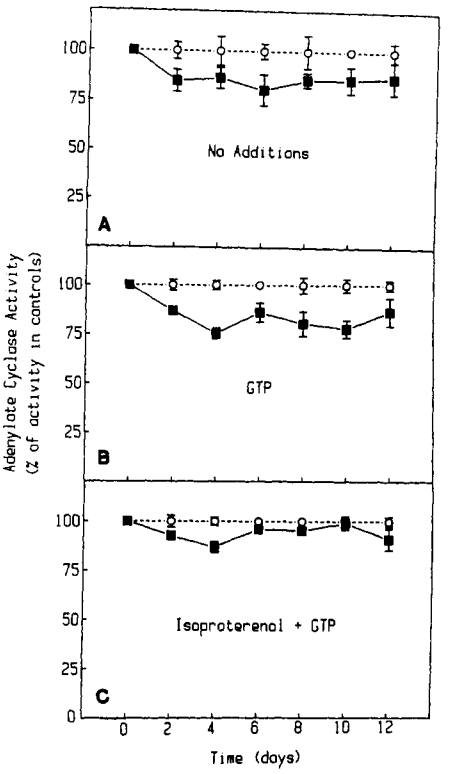

FIGURE 1: Effect on adenylate cyclase activity of exposure of S49 cells to 50 mM ethanol for different lengths of time. Adenylate cyclase activity was measured in membranes prepared from S49 cells after growth in the absence (○) or presence (■) of 50 mM ethanol for the indicated lengths of time. Activity was measured in the presence of no additions (A), GTP (B), or isoproterenol plus GTP (C). The data are expressed as the percent of the average activity determined on the same day in membranes prepared from control cells. The data are the means ± SEM of the activity in membranes prepared from between 3 and 23 separate cultures assayed on between 1 and 9 different days, each of which was assayed in duplicate. For each graph, the activity in membranes prepared from control cells was compared with the activity in membranes prepared from ethanol-treated cells by two-way analysis of variance. In each case, the ethanol-treated group was significantly different from the control group, and there was no significant effect of time of exposure. The levels of significance for each activity were the following: no additions, $p < 0.01$; GTP, $p < 0.001$; isoproterenol plus GTP, $p < 0.01$.

The effect on adenylate cyclase activity of chronic exposure of S49 cells to ethanol was determined (Figure 1). Exposure of S49 lymphoma cells to 50 mM ethanol for different lengths of time resulted in a decrease in the activity of adenylate cyclase measured without any additions (Figure 1A) or in the presence of GTP (Figure 1B). The activity measured in the presence of isoproterenol plus GTP was less sensitive to chronic treatment with ethanol (Figure 1C). A maximal effect was seen after growing cells in the presence of ethanol for 2–4 days. Two-way analysis of variance indicated that the effect of exposure time was not statistically significant.

Experiments were carried out to determine whether ethanol had effects on the physical properties of the membrane. The steady-state anisotropy of DPH in membranes prepared from S49 cells that had been treated with ethanol was significantly lower (i.e., membrane fluidity was higher) than the anisotropy in membranes prepared from control cells (Figure 2).

If the acute effect of ethanol in vitro on membrane fluidity is important in producing the decrease in adenylate cyclase activity after chronic treatment with ethanol, then long-term exposure to a different treatment that acutely increases membrane fluidity should also lead to a decrease in adenylate cyclase activity. Membrane fluidity increases with increasing temperature (Harris & Schroeder, 1981). GTP-stimulated

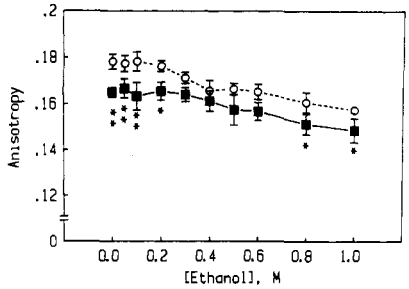

FIGURE 2: Effect of ethanol on fluorescence anisotropy in membranes prepared from S49 cells after exposure to 50 mM ethanol for 4 days. The steady-state fluorescence anisotropy of DPH was determined in the presence of increasing concentrations of ethanol in vitro, using purified plasma membranes prepared from S49 cells grown in the absence (○) or presence (■) of 50 mM ethanol for 4 days. Anisotropy was measured at 37 °C. The data are the means ± SEM of the anisotropy values determined with membranes prepared from three separate cultures on 3 different days. Each point on the curve for the ethanol-treated group was compared to the corresponding point on the control curve. Statistical analysis was performed using a two-way analysis of variance for blocked data (one asterisk, $p < 0.05$; two asterisks, $p < 0.01$).

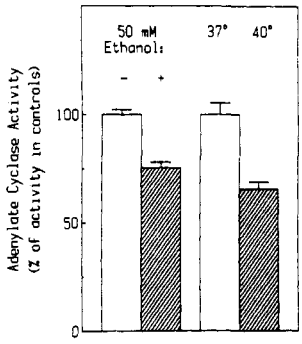

FIGURE 3: Effect on adenylate cyclase activity of chronic exposure of S49 cells to ethanol or elevated temperature. Adenylate cyclase activity was measured in membranes prepared from S49 cells following growth for 4 days in the absence (open bar, left) or presence (hatched bar, left) of 50 mM ethanol, or at 37 °C (open bar, right) or 40 °C (hatched bar, right) for 8 days. Activity was measured in the presence of 50 μM GTP. The data are expressed as a percent of the average activity determined on the same day using membranes prepared from cells grown under control conditions (at 37 °C in the absence of alcohol). The data are the means ± SEM of the activity in membranes prepared from 3–23 separate cultures assayed on 1–9 different days, each of which was assayed in duplicate. A t test on each pair of bars was used to determine that the effect of each treatment was statistically significant (ethanol, $p < 0.001$; elevated temperature, $p < 0.01$).

adenylate cyclase activity was lower in membranes prepared from cells that had been grown at 40 °C for 8 days than in membranes from cells that were grown at 37 °C (Figure 3). Although growth of S49 cells at 40 °C resulted in a change in adenylate cyclase activity similar to the change that was seen following chronic exposure to 50 mM ethanol (Figure 3), this treatment had no effect on membrane fluidity, as determined by the fluorescence anisotropy of DPH (data not shown).

Experiments were carried out to determine whether the decrease in adenylate cyclase activity after chronic exposure to ethanol was accompanied by changes in the properties of β-adrenergic receptors. The densities of β-adrenergic receptors and the affinities of the receptors for [^{125}I]IPIN were nearly identical in membranes prepared from control cells and in membranes prepared from cells that had been treated with 50 mM ethanol for 4 days. In addition, there was no difference in the effect of ethanol in vitro on the binding of [^{125}I]IPIN in membranes prepared from control and ethanol-treated cells (Figure 4). The inhibition of the binding of [^{125}I]IPIN by

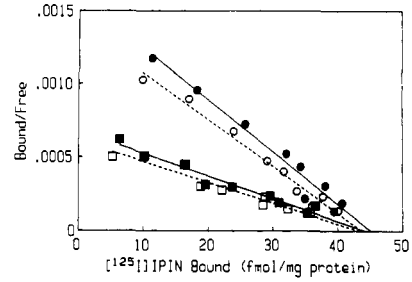

FIGURE 4: Effect on the binding of [^{125}I]IPIN of exposure of S49 cells to 50 mM ethanol for 4 days. The binding of [^{125}I]IPIN was determined in the absence (○, ●) or presence (□, ■) of 0.5 M ethanol, using membranes prepared from S49 cells after growth in the absence (○, □) or presence (●, ■) of 50 mM ethanol for 4 days. The specific binding of [^{125}I]IPIN was analyzed by the method of Scatchard (1949). The data shown are the means of duplicate determinations and are representative of data obtained in up to seven independent experiments. The K_d values in membranes prepared from control cells were 32.0 × 10^{-12} and 73.9 × 10^{-12} M in the absence and presence, respectively, of 0.5 M ethanol in vitro. The corresponding K_d values for membranes prepared from ethanol-treated cells were 32.3 × 10^{-12} and 70.9 × 10^{-12} M. The B_{max} values (in femtomoles per milligram of protein) for membranes from control cells were 42.8 and 43.4 in the absence and presence of 0.5 M ethanol in vitro and 46.8 and 45.9 for membranes from ethanol-treated cells.

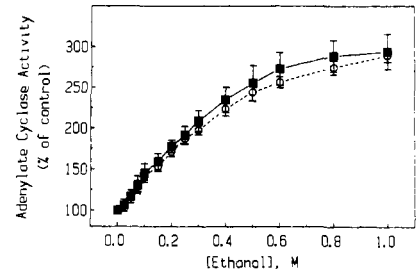

FIGURE 5: Effect of ethanol in vitro on adenylate cyclase activity in membranes prepared from control and ethanol-treated S49 cells. Adenylate cyclase activity was measured in the presence of increasing concentrations of ethanol using membranes prepared from S49 cells grown in the absence (○) or presence (■) of 50 mM ethanol for 4 days. Assays contained 50 μM GTP. The data are expressed as a percent of the activity measured in the absence of ethanol and represent the mean ± SEM of the activity in membranes prepared from three separate cultures, each of which was assayed in duplicate. The activities (picomoles of cAMP per minute per milligram of protein) measured in the absence of ethanol (mean ± SEM) were 4.74 ± 0.49 (n = 23) in control membranes and 3.50 ± 0.44 (n = 21) in membranes prepared from ethanol-treated cells.

isoproterenol, in the absence or presence of GTP, was the same in membranes prepared from control and from treated cells (data not shown).

Addition of ethanol in vitro resulted in an increase in adenylate cyclase activity in membranes prepared from S49 cells (Figure 5; Bode & Molinoff, 1988). The basal activity (no additions) as well as activities measured in the presence of GTP and in the presence of isoproterenol plus GTP were increased by ethanol in vitro. There were no differences between membranes prepared from control and from ethanol-treated cells in terms of the effects of ethanol in vitro on adenylate cyclase activity. This was true in the presence (Figure 5) or absence (data not shown) of GTP or in the presence of isoproterenol plus GTP (data not shown). Furthermore, addition of ethanol in vitro resulted in an increase in the EC_{50} for stimulation of adenylate cyclase activity by isoproterenol in membranes prepared from S49 cells after chronic treatment with ethanol (data not shown), just as it did in membranes prepared from control cells (Bode & Molinoff, 1988). Thus, there was no evidence for tolerance to the acute, in vitro effects of ethanol on the β-adrenergic receptor-coupled adenylate

Table I: Effect of Exposure to 50 mM Ethanol for 4 Days on the Phospholipid Composition and Cholesterol Content of Plasma Membranes Prepared from S49 Cells[a]

	control	ethanol treated
phosphatidic acid (%)	2.8 ± 0.2	2.9 ± 0.4
phosphatidylcholine + phosphatidylinositol (%)	60.1 ± 0.1	59.1 ± 0.4
phosphatidylserine (%)	11.0 ± 0.1	9.6 ± 0.3*
cardiolipin (%)	5.6 ± 0.3	6.3 ± 0.2
phosphatidylethanolamine (%)	20.5 ± 0.1	22.0 ± 0.2**
μmol of cholesterol/mg of protein	0.164 ± 0.004	0.165 ± 0.014
μmol of phospholipid/mg of protein	0.548 ± 0.011	0.555 ± 0.014
cholesterol:phospholipid molar ratio	0.300 ± 0.007	0.296 ± 0.021

[a] The data (expressed as the percent by weight of each phospholipid) represent the means ± SEM determined from three separate cultures in each group. Statistical analysis was carried out by performing a t test on each control/ethanol-treated pair (one asterisk, $p < 0.05$; two asterisks, $p < 0.01$). The "cardiolipin" band was identified by comparison of its retention time in one-dimensional thin-layer chromatography with an authentic cardiolipin standard.

Table II: Effect of Exposure to 50 mM Ethanol for 4 Days on the Fatty Acid Composition of Plasma Membranes Prepared from S49 Cells[a]

fatty acid	control (%)	ethanol treated (%)
12:0	0.1 ± 0.0	0.1 ± 0.0
14:0	3.1 ± 0.2	3.3 ± 0.4
16:0	31.3 ± 2.9	34.0 ± 2.4
16:1	2.5 ± 0.5	1.6 ± 0.7
18:0	15.7 ± 1.5	17.0 ± 1.7
18:1	14.1 ± 1.9	12.4 ± 2.4
18:2	16.9 ± 3.7	11.8 ± 3.8
18:3	4.5 ± 1.5	2.6 ± 1.3
18:4	6.9 ± 3.5	12.6 ± 3.9
20:4	1.1 ± 0.2	0.9 ± 0.2
22:6	0.4 ± 0.2	0.4 ± 0.2
24:0	1.6 ± 0.2	1.6 ± 0.2
24:1	1.8 ± 0.1	1.9 ± 0.2

[a] The data are expressed as the percent by weight of each fatty acid methyl ester and represent the means ± SEM determined from three separate cultures in each group. Each control/ethanol-treated pair was compared statistically by performing a t test. No significant differences were found.

cyclase system after chronic exposure of S49 cells to ethanol.

The lipid composition of plasma membranes prepared from S49 cells was determined after exposure to 50 mM ethanol for 4 days. Membranes from control and from ethanol-treated cells showed no differences in the cholesterol content, phospholipid content, or the cholesterol to phospholipid ratio (Table I). Quantitative analysis of individual phospholipids revealed a small but statistically significant decrease in the proportion of phosphatidylserine and a small but statistically significant increase in the proportion of phosphatidylethanolamine after chronic exposure to ethanol (Table I). Ethanol treatment did not significantly affect the proportions of the other phospholipids.

The fatty acid composition of the plasma membrane was unchanged after exposure of S49 cells to 50 mM ethanol for 4 days. Membranes prepared from control and from ethanol-treated cells showed no differences in the proportions of any of the fatty acids (Table II).

Discussion

The alterations in the physical and functional properties of the plasma membrane that occur after chronic exposure of S49 cells to ethanol can be analyzed in the context of the Hill and Bangham (1975) model of tolerance to and dependence on general anesthetics. More specifically, it can be asked whether the changes in the functional properties of the membrane after chronic treatment with ethanol are related to changes in the physical properties of the membrane. The utility of the β-adrenergic receptor-coupled adenylate cyclase system for this analysis derives from the fact that it is a transmembrane, multicomponent system. Thus, the functional properties of the proteins may be sensitive to changes in the physical properties of the membrane. According to the Hill and Bangham hypothesis, tolerance to ethanol would be reflected in a decrease in the ability of ethanol in vitro to increase membrane fluidity. Dependence would be expected to result from a decrease in the fluidity of the membrane, measured in the absence of ethanol in vitro. In terms of the effects of ethanol on the functional properties of the membrane, tolerance would be associated with a reduction in the effects of ethanol in vitro on the β-adrenergic receptor-coupled adenylate cyclase system. A change observed after chronic exposure to ethanol that is opposite to the effect of ethanol in vitro might be a sign of dependence.

Tolerance to the in vitro effects of ethanol was not demonstrated by changes in any of the indices of the physical or functional properties of the membrane that were measured in these studies. Thus, addition of ethanol in vitro had the same effect on fluorescence anisotropy in membranes prepared from control cells as in membranes prepared from ethanol-treated cells. In addition, the effects of ethanol in vitro on the binding of [^{125}I]IPIN to β-adrenergic receptors were the same in membranes prepared from control and from ethanol-treated cells. There were no differences between control and treated cells in terms of the stimulation of adenylate cyclase activity by ethanol in vitro or the effect of ethanol in vitro on the EC_{50} value for stimulation of adenylate cyclase activity by isoproterenol.

The activity of adenylate cyclase was lower in membranes prepared from ethanol-treated S49 cells than in membranes prepared from control cells. This change could be the result of an adaptive response leading to dependence, since the change in activity after chronic treatment was the opposite of the acute effect of ethanol. The major effect observed after chronic treatment of S49 cells with ethanol was a decrease in adenylate cyclase activity measured in the presence of GTP. These findings are consistent with the observation that, in membranes prepared from S49 cells, the stimulation by ethanol in vitro of the activity measured in the presence of isoproterenol plus GTP is due entirely to stimulation of the activity measured in the presence of GTP alone (Bode & Molinoff, 1988). The observation that a maximal effect on adenylate cyclase activity was seen after exposure to ethanol for 2–4 days might be expected, based on the 16-h doubling time of S49 cells.

Changes in the density of particular classes of receptors in selected brain regions have been observed after chronic treatment of animals with ethanol (Tabakoff et al., 1979; Rabin et al., 1980; Lucchi et al., 1984). In the present studies, no changes were detected in the properties of the β-adrenergic receptors after chronic exposure of cells to ethanol. Thus, the density of β-adrenergic receptors, the affinity of the receptors for [^{125}I]IPIN and isoproterenol, and the effect of GTP on the affinity of the receptors for isoproterenol were the same in membranes prepared from control and from ethanol-treated cells.

In contrast to the effects of chronic treatment with ethanol on the functional properties of the membrane as indicated by the decrease in adenylate cyclase activity, the effects of chronic exposure to ethanol on the physical properties of the membrane in terms of an adaptive response to ethanol are more difficult to explain. The acute effect of ethanol is to decrease an-

isotropy, so an adaptive response leading to dependence would be expected to result in an increase in anisotropy. Thus, the decrease in anisotropy observed after chronic exposure of S49 cells to ethanol is not consistent with the Hill and Bangham model for the development of dependence on ethanol.

The decrease in anisotropy could be due to residual ethanol in the membranes. This is unlikely, because the measurements of fluorescence anisotropy were made with a purified plasma membrane preparation that had been washed extensively. In any case, the concentration of ethanol (50 mM) used to treat the cells was too low to account for the difference in anisotropy between the control and treated cells (Figure 2). Polokoff et al. (1985) observed a similar decrease in anisotropy after chronic exposure of cultured Reuber H35 hepatoma cells to ethanol. However, their results appeared to be due to the production of acetaldehyde by the cells. This explanation cannot account for the results presented here, because S49 cells do not metabolize significant amounts of ethanol to acetaldehyde, although the cells are capable of metabolizing acetaldehyde. This is an important observation in view of the lack of production of $^{14}CO_2$ from [^{14}C]ethanol. If [^{14}C]-acetaldehyde had been produced, it would have been further metabolized and would have appeared as $^{14}CO_2$.

The decrease in anisotropy observed after chronic exposure to ethanol was apparently not due to changes in the lipid composition of the plasma membrane. It cannot be explained by an alteration in the ratio of saturated to unsaturated fatty acids, because there were no significant differences in the proportions of any of the fatty acids in membranes from control and from ethanol-treated cells. Similarly, it cannot be explained by a decrease in the cholesterol to phospholipid ratio, because there was no change in this parameter after chronic exposure to ethanol. The only changes in the lipid composition of the plasma membrane were a small decrease in the proportion of phosphatidylserine and a small increase in the proportion of phosphatidylethanolamine. The relationship, if any, between the amount of phosphatidylserine and membrane fluidity is unknown. An increase in the amount of phosphatidylethanolamine would not be expected to cause a decrease in anisotropy. In fact, an increase in phosphatidylethanolamine has been reported to be associated with a decrease in membrane fluidity (Esko et al., 1977; Gilmore et al., 1979), which is equivalent to an increase in anisotropy.

It is possible that changes in the lipid composition of the plasma membrane occurred that were not detected in these studies. Changes in the fatty acid composition of individual phospholipids, such as those reported by Harris et al. (1984a) in rats after chronic treatment with ethanol, would be difficult to detect in cultured cells due to the difficulty of obtaining a sufficient amount of tissue. Acyl and alkyl chains derived from ether lipids (plasmalogens) are included with fatty acids derived from the other phospholipids, so changes in the composition of these chains would not be detected. Gangliosides have been reported to enhance the effect of ethanol on anisotropy in vitro (Harris et al., 1984b). Changes in the amount or distribution of gangliosides, or in the composition of their hydrocarbon chains, after chronic exposure to ethanol might affect membrane fluidity but would not be detected by the methods used in these studies.

It is also possible that alterations in the structure of the membrane that are unrelated to the lipid composition occur after chronic exposure to ethanol. The anisotropy of DPH, which appears to reflect the order of all regions of the membrane at an intermediate depth in the bilayer, would probably not detect localized alterations in anisotropy. Changes in the proportions of solid or fluid domains may occur after chronic exposure to ethanol. The use of probes such as *cis*- and *trans*-parinaric acid, which partition differentially into solid and fluid lipid regions, might make it possible to detect such changes. To investigate the possibility of differential alterations in anisotropy at different depths in the bilayer, results obtained with probes of the surface could be compared with those obtained with probes of the interior of the bilayer. Changes in membrane proteins may be responsible for part of the decrease in anisotropy after chronic exposure to ethanol. This could be investigated by determining the anisotropy of protein-free lipid extracts.

The alterations in membrane fluidity and adenylate cyclase activity observed after chronic treatment with ethanol suggest a possible relationship between membrane fluidity and adenylate cyclase activity. In most cases, treatments that increase membrane fluidity are associated with increased enzyme activity. However, the stimulation of adenylate cyclase activity by alcohols is not simply a consequence of an increase in bulk membrane fluidity (Rabin et al., 1986). Furthermore, there are instances in which decreased membrane fluidity is associated with increased activity [e.g., see Sinensky et al. (1979)]. In the present studies, exposure to increased temperature resulted in a decrease in adenylate cyclase activity but had no effect on fluorescence anisotropy. This suggests that the decrease in anisotropy after chronic treatment with ethanol is not responsible for the decrease in adenylate cyclase activity and, in fact, there may be no relationship between changes in membrane fluidity and changes in adenylate cyclase activity.

In summary, these results suggest that the changes in the physical and functional properties of the membrane observed after chronic treatment with ethanol may be unrelated. There was no evidence for tolerance to the in vitro effects of ethanol. In the context of the Hill and Bangham hypothesis, the observed change in membrane fluidity is not consistent with an adaptive response leading to dependence on ethanol.

Registry No. Ethanol, 64-17-5.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Bode, D. C., & Molinoff, P. B. (1988) *J. Pharmacol. Exp. Ther.* (in press).

Charness, M. E., Gordon, A. S., & Diamond, I. (1983) *Science (Washington, D.C.) 222*, 1246–1248.

Chin, J. H., & Goldstein, D. B. (1977) *Science (Washington, D.C.) 196*, 684–685.

Chin, J. H., Parsons, L. M., & Goldstein, D. B. (1978) *Biochim. Biophys. Acta 513*, 358–363.

Dugan, E. A. (1985) *LC 3*, 126–128.

Eriksson, C. J. P., Sippel, H. W., & Forsander, O. A. (1977) *Anal. Biochem. 80*, 116–124.

Esko, J. D., Gilmore, J. R., & Glaser, M. (1977) *Biochemistry 16*, 1881–1890.

Gilmore, R., Cohn, N., & Glaser, M. (1979) *Biochemistry 18*, 1042–1049.

Gordon, A. S., Collier, K., & Diamond, I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2105–2108.

Harris, R. A., & Schroeder, F. (1981) *Mol. Pharmacol. 20*, 128–137.

Harris, R. A., Baxter, D. M., Mitchell, M. A., & Hitzemann, R. J. (1984a) *Mol. Pharmacol. 25*, 401–409.

Harris, R. A., Groh, G. I., Baxter, D. M., & Hitzemann, R. J. (1984b) *Mol. Pharmacol. 25*, 410–417.

Hill, M. W., & Bangham, A. D. (1975) in *Alcohol Intoxication and Withdrawal II* (Gross, M. M., Ed.) pp 1–9, Plenum, New York.
Hoffman, P. L., & Tabakoff, B. (1977) *Nature (London) 268*, 551–553.
Ingram, L. O. (1976) *J. Bacteriol. 125*, 670–678.
Ingram, L. O., Ley, K. D., & Hoffmann, E. M. (1978) *Life Sci. 22*, 489–494.
Keegan, R., Wilce, P. A., Ruczkal-Pietrzak, E., & Shanley, B. C. (1983) *Biochem. Biophys. Res. Commun. 114*, 985–990.
Koerker, R. L., Berlin, A. J., & Schneider, F. H. (1976) *Toxicol. Appl. Pharmacol. 37*, 281–288.
Levental, M., & Tabakoff, B. (1980) *J. Pharmacol. Exp. Ther. 212*, 315–319.
Littleton, J. M., & John, G. (1977) *J. Pharm. Pharmacol. 29*, 579–580.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lucchi, L., Rius, R. A., Uzumaki, H., Govoni, S., & Trabucchi, M. (1984) *Brain Res. 293*, 368–371.
Lyon, R. C., & Goldstein, D. B. (1983) *Mol. Pharmacol. 23*, 86–91.
Minneman, K. P., Hegstrand, L. R., & Molinoff, P. B. (1979) *Mol. Pharmacol. 16*, 21–33.
Morrison, W. R., & Smith, L. M. (1964) *J. Lipid Res. 5*, 600–608.
Morrisson, M., Wilce, P. A., & Shanley, B. C. (1984) *Biochem. Biophys. Res. Commun. 122*, 516–521.
Nandini-Kishore, S. G., Mattox, S. M., Martin, C. E., & Thompson, G. A., Jr. (1979) *Biochim. Biophys. Acta 551*, 315–327.
Polokoff, M. A., Simon, T. J., Harris, R. A., Simon, F. R., & Iwahashi, M. (1985) *Biochemistry 24*, 3114–3120.
Rabin, R. A., Wolfe, B. B., Dibner, M. D., Zahniser, N. R., Melchior, C., & Molinoff, P. B. (1980) *J. Pharmacol. Exp. Ther. 213*, 491–496.
Rabin, R. A., Bode, D. C., & Molinoff, P. B. (1986) *Biochem. Pharmacol. 35*, 2331–2335.
Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5761–5775.
Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Sinensky, M. (1980) *J. Cell Biol. 85*, 166–169.
Sinensky, M., Minneman, K. P., & Molinoff, P. B. (1979) *J. Biol. Chem. 254*, 9135–9141.
Stenstrom, S., & Richelson, E. (1982) *J. Pharmacol. Exp. Ther. 221*, 334–341.
Syapin, P. J., & Noble, E. P. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E. P., Eds.) Vol. 1, pp 521–540, Plenum, New York.
Tabakoff, B., Munoz-Marcus, M., & Fields, J. Z. (1979) *Life Sci. 25*, 2173–2180.
Walker, F., Elmslie, W., Fraser, R. A., Snape, P. E., & Watt, G. C. M. (1974) *Scott. Med. J. 19*, 125–127.
Wolfe, B. B., & Harden, T. K. (1981) *J. Cyclic Nucleotide Res. 7*, 303–312.

Specificity of the Sarcoplasmic Reticulum Calcium ATPase at the Hydrolysis Step†

David M. Chipman*,‡ and William P. Jencks*
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254
Received January 12, 1988; Revised Manuscript Received March 25, 1988

ABSTRACT: The coupling of Ca^{2+} transport to ATP hydrolysis by the SR ATPase requires that the enzyme operate with considerable specificity, which is different at different steps. The limits of specificity of the calcium-free phosphorylated enzyme for transfer of its phosphoryl group to water have been examined. The rate of transfer of the phosphoryl group to the simple nucleophile methanol was compared to its transfer to water by following the formation of methyl phosphate from inorganic phosphate. The reverse reaction, hydrolysis of methyl phosphate, was compared to phosphate–water oxygen exchange. The reactions involving methanol as nucleophile or leaving group are at least 2–3 orders of magnitude slower than those involving water. This result indicates that the transition state for this reaction involves strong and specific interactions of the H_2O molecule with the enzyme. These interactions may also involve the bound Mg^{2+} ion. The results also suggest that the difference in specificity between Ca^{2+} free and Ca^{2+} bound states of the enzyme involves significant differences in the structure of the catalytic site.

The Ca^{2+}-ATPase of sarcoplasmic reticulum is one of the most thoroughly studied coupled membrane ion pumps; considerable progress has been made toward an understanding of the mechanism by which it mediates the interconversion of chemical energy and osmotic work (de Meis & Vianna, 1979; Ikemoto, 1982; Martonosi & Beeler, 1983; Inesi, 1985). A large body of experimental evidence has been gathered in support of a stepwise reaction cycle for the enzyme (Scheme I) that can explain the stoichiometric coupling of the vectorial transport of calcium ions across the SR[1] membrane to the hydrolysis of ATP. This reaction scheme (like other related

† This research was supported in part by grants to W.P.J. from the National Institutes of Health (GM20888) and the National Science Foundation (PCM81-17816). A preliminary report of this work was presented at the Ion Pump Workshop, Jerusalem, Aug 30–Sept 2, 1987.
‡ Department of Biology, Ben Gurion University of the Negev, Beer Sheva, Israel.

[1] Abbreviations: SR, sarcoplasmic reticulum; E, calcium ATPase; EP, phosphorylated calcium ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MeOP, methyl phosphate; BSA, bovine serum albumin.

0006-2960/88/0427-5707$01.50/0