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# EFFECT OF ETHANOL ON PHOSPHOLIPID TURNOVER AND CALCIUM MOBILIZATION IN CHICK EMBRYOS

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**Abstract**—Effects of ethanol on [$^3$H]inositol and [$^{14}$C]choline incorporation into phosphatidylinositol (PI) and phosphatidylcholine (PC), free intrasynaptosomal $Ca^{2+}$ ($[Ca^{2+}]_i$) and synaptosomal $^{45}Ca^{2+}$ uptake, were investigated in the brain and heart of 17-day-old chick embryos to which a 10% ethanol solution had been injected on the 3rd day of embryogenesis. In brain synaptosomes, ethanol increased the incorporation of [$^3$H]inositol and [$^{14}$C]choline into PI and PC, increased $[Ca^{2+}]_i$, and decreased $^{45}Ca^{2+}$ uptake. On the other hand, in heart synaptosomal membrane, ethanol decreased the incorporation of [$^3$H]inositol and [$^{14}$C]choline into PI and PC, decreased $[Ca^{2+}]_i$, and increased $^{45}Ca^{2+}$ uptake. Ethanol stimulated *in vitro* [$^3$H]inositol and [$^{14}$C]choline incorporation into PI and PC in the brain and heart in both the control and ethanol-treated groups. However, addition of ethanol did not affect the release of $^{45}Ca^{2+}$ from the synaptosomal membrane of either organ in either group. Addition of ethanol inhibited $^{45}Ca^{2+}$ uptake in a dose-dependent manner in the brain but not in the heart. In both organs, there was a relationship between phospholipid turnover and $[Ca^{2+}]_i$ after ethanol.

Chin and Goldstein [1] reported that ethanol at pharmacological concentrations can fluidize biological membranes, and that this effect diminishes with chronic exposure. Hill and Bangham [2] proposed that general anesthetic such as alcohol increases $K^+$ permeability of liposomes in a protein-free system at anesthetic concentrations. Membrane tolerance was observed in multilamellar vesicles composed of phospholipids extracted from membranes of synaptosomes [3], hepatic mitochondria [4] and microsomes [5, 6] from ethanol-treated animals. Thus, tolerance is clearly a property of the phospholipid portion of the membrane. Despite numerous studies of the effects of chronic ethanol consumption on the lipid composition of membranes, no larger changes have been reported and no consistent pattern has emerged. Thus, the molecular basis of membrane tolerance remains obscure.

The importance of calcium ($Ca^{2+}$) in the activity of excitable membrane has been well established. A phospholipid, phosphatidylserine, is likely to be involved in $Ca^{2+}$ binding, since it has been shown to have a net negative charge at neutral pH [7, 8]. Ross *et al.* [9] found a selective depletion of brain $Ca^{2+}$ induced by morphine and ethanol. The mechanism is not understood but a fundamental event in membrane excitation is dissociation of $Ca^{2+}$ from negatively charged binding sites of phospholipids with high affinity for divalent cations. It has been reported that ethanol antagonizes the release of acetylcholine from both whole brains and from cortex slices [10], and that ethanol decreases brain $Ca^{2+}$ [9], which plays an important role in transmitter release and neuronal activity.

This study examined the effects of ethanol on the incorporation of [$^3$H]inositol and [$^{14}$C]choline into PI and PC in synaptic fractions, with continuous exposure of the chick embryo brain and heart to ethanol [11]. The labeled phospholipids were followed as indicators of the changes induced by ethanol in the turnover of a membrane component. This is assumed to reflect altered physical states of the membranes and their $Ca^{2+}$ binding capacity.

## MATERIALS AND METHODS

Fertile eggs of a White Leghorn strain were obtained from a local hatchery (Shimizu Lab. Supplies Co., Ltd, Kyoto, Japan). Eggs were set in a rotating forced air incubator (Masterpiece Elect. Incubators Model No. F250, Shibata Incubator Manufacture Co., Hiroshima, Japan), maintained at 37° and 90% relative humidity, rotating every 8 hr. Fetal chicks were chronically treated with ethanol by injection of a 10% ethanol solution into the air sac on day 3 of embryogenesis as described previously [11]. In this method, ethanol was detected in the yolk or albumen on day 10 of incubation. However, some of the ethanol in the embryo may have been metabolized.

*Preparation of synaptosomes.* Both vehicle and ethanol treated embryos were decapitated as previously reported [11]. The brain (without the cerebellum) and heart were excised quickly and put on ice. Synaptosomes were prepared by Dodd's [12] modification of the method of Gray and Whittaker [13]. Data were expressed from five or six embryos in each paired group. Briefly, after homogenization in 10 volumes of cold 0.32 M sucrose, the homogenate was centrifuged (1500 *g*, 10 min, 0–4°) and the supernatant was laid over 1.2 M sucrose. After centrifugation (50,000 rpm, 10 min; Kontron R.

model TGA-65, rotor TST54), the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and laid over 0.8 M sucrose. After centrifugation again at high speed the synaptosomal pellet was suspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., MO, U.S.A.) buffer (pH 7.4) and was then used in [$^3$H]inositol and [$^{14}$C]choline incorporation, $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake studies. The composition of HEPES buffer was (millimolar): NaCl, 125; KCl, 5; $NaH_2PO_4$, 1.2: $NaHCO_3$, 5; glucose, 6; $CaCl_2$, 1; and HEPES, 25.

*Assay of* [$^3$H]*inositol and* [$^{14}$C]*choline incorporation into phospholipids.* Synaptosomal suspensions were added to both HEPES buffer and a sodium-free medium (pH 7.4) containing [$^3$H]inositol and [$^{14}$C]choline. The composition of the $Na^+$-free medium was identical except that NaCl, $NaHCO_3$ and $NaH_2PO_4$ were replaced by sucrose (260 mM) and Tris phosphate (1.2 mM). The assay was done as described by Porcellati and colleagues [14]. The reaction mixture contained about 600 μg of protein and 0.5 μCi of [$^3$H]inositol (*myo*-[2-$^3$H(*N*)]inositol, sp. act. 17.10 Ci/mmol, New England Nuclear, MA, U.S.A.) and 0.1 μCi of [$^{14}$C]choline ([1,2-$^{14}$C]choline chloride, sp. act. 7.2 mCi/mmol, New England Nuclear) in 1 mL of the medium. The incubation continued for 15 min at 37°. It was terminated by adding 6 mL of $CHCl_3$–MeOH (2:1, v/v), and mixing with 0.2 mL of 0.17 M KCl including 10 mM inositol and choline. The radiolabeled phospholipids were extracted as described by Eichberg and Dawson [15], the extracts were evaporated under vacuum and redissolved in a small volume of chloroform and an aliquot was applied to a 250 μm silica gel G thin-layer chromatography plate (TLC). The plate was developed in a solvent system of chloroform, methanol, concentrated acetic acid and water (25:15:4:2, by vol.). After development, [$^3$H]PI and [$^{14}$C]PC specific activities were determined as described elsewhere [16]. Lipid phosphorus levels were analysed as described by Bartlett [17]. Data are presented as dpm/g protein of incubation medium or dpm/μmol of phospholipid-P applied to the plate.

*Assay of* $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ were determined by a modification of the method of Komulainen and Bondy [18] using fura-2/AM (Dojin Co. Inc., Kumamoto, Japan). Briefly, 1 mL aliquots of the synaptosome suspension (600 μg of protein) were incubated with 1 μM fura- 2/AM in DMSO (final concentration, 1%) at 37° for 20 min and then centrifuged (13,000 *g* for 30 sec). The pellets were resuspended in 1 mL HEPES buffer which did not contain $NaHCO_3$ or $NaH_2PO_4$ in order to prevent precipitation of high levels of $Ca^{2+}$. The emitted fluorescence of the sample was measured in a 4° cuvette using a fluorescence spectrophotometer (model F-3010, Hitachi, Co. Inc., Tokyo, Japan) at excitation wavelengths of 340 and 380 nm (bandpass, 1 nm) and at 510 nm for emission (bandpass, 8 nm). Mixing was carried out with a vortex mixer 30 sec before reading fluorescence. $[Ca^{2+}]_i$ was calculated using the formula of Grynkiewicz *et al.* [19]:

$$[Ca^{2+}]_i = K_d \times (R - R_{min})\ (Sf_2)/(R_{max} - R)\ (Sb_2).$$

For calibration of the synaptosomal fura-2–$Ca^{2+}$ signal ($R$), $R_{min}$ (the ratio of fluorescence at 340 nm/380 nm in the absence of $Ca^{2+}$) and $R_{max}$ (the ratio when all fura-2 of the sample was saturated with $Ca^{2+}$) were determined for each batch of fura-2-loading synaptosomes where $K_d$ (224 nM) is the dissociation constant of fura-2–$Ca^{2+}$ complex, and $Sf_2$ and $Sb_2$ denote fluorescence of fura-2 at zero $Ca^{2+}$ and $Ca^{2+}$ saturation, respectively, at the excitation wavelength 380 nm.

*Assay of* $^{45}Ca^{2+}$ *uptake.* Synaptosomal calcium uptake was studied according to the method of Blaustein and Weismann [20]. Synaptosomal suspensions in BSS (buffered salt solution) were brought to a final tissue concentration of 0.5–0.8 mg protein/mL. The composition of BSS was (millimolar): NaCl, 120; KCl, 5; $NaH_2PO_4$, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.0; glucose, 10; HEPES, 20 (pH 7.5). Aliquots (1.0 mL) of the suspension were transferred to 12 mL polypropylene centrifuge tubes and preincubated for 3 min at 37°. Test ethanol was added in 20 μL volumes at the beginning of the equilibration period. $^{45}Ca^{2+}$ Uptake was initiated with the addition of 1.0 mL of prewarmed (20°) BSS containing 0.5 μCi of $^{45}Ca^{2+}$ ($^{45}CaCl_2$, sp. act. 20.08 mCi/mg, New England Nuclear). The $^{45}Ca^{2+}$ uptake incubations were terminated 10 min after the addition of the radiotracer by adding 3.0 mL of ice-cold $Mg^{2+}$, $Ca^{2+}$-free BSS containing 3.0 mM of the calcium chelator EGTA (ethyleneglycol-bis[β-aminoethyl ether]-*N*,*N'*-tetraacetic acid). Incubation mixtures were immediately centrifuged (18,000 *g* for 4 min), and synaptosomal pellets were washed once in ice-cold $Ca^{2+}$-free BSS. The final pellets were digested in 0.5 mL of 0.5 N NaOH by heating to 50° for 30 min. Following neutralization with 0.5 N HCl, the samples were added to 10 mL aqueous counting scintillant (ACS II, Amersham, IL, U.S.A.) for determination of the synaptosomal $^{45}Ca^{2+}$ content by liquid scintillation spectrometry (LSC-9000, Aloka, Tokyo, Japan).

*Synaptosomal* $^{45}Ca^{2+}$ *efflux.* To study the spontaneous efflux of calcium from the synaptosomes, the protocol for $^{45}Ca^{2+}$ uptake was modified. Synaptosomes suspended in BSS at a concentration of 10–15 mg protein/mL were preloaded with the radiotracer by incubation at 20° for 60 min in the presence of 10 μCi $^{45}Ca^{2+}$/mL. The specific activity of this radioactive incubation medium was measured to determine the absolute values (nmol $Ca^{2+}$) released in subsequent incubations. The loading incubations were terminated with the addition of three volumes of ice-cold BSS. After centrifugation (18,000 *g* for 4 min), the radioactive supernatant was discarded. Two washes in ice-cold BSS preceded the final resuspension of synaptosomal pellets in BSS at a final tissue concentration of 0.5–0.8 mg/mL. Aliquots (1.0 mL) of the $^{45}Ca^{2+}$-labeled synaptosomal suspension were transferred to 12 mL polypropylene centrifuge tubes for a 2 min preincubation in the presence or absence of ethanol. The 10 min incubation during which efflux was measured was initiated with the addition of 1.0 mL prewarmed BSS (22°). Incubation was terminated after 10 min by the addition of ice-cold BSS and the suspension was immediately centrifuged. Aliquots

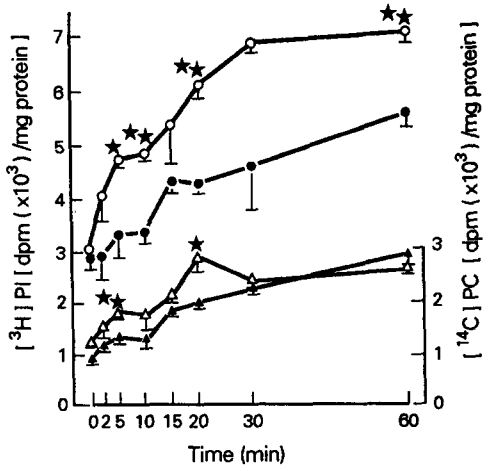


Fig. 1. Time course of changes on inositol and choline incorporation into chick brain synaptosomal phospholipids. The isolated synaptosomes from 17-day-old chick embryo brains which had received a 10% solution of ethanol or saline in the air sac on day 3 of embryogenesis were incubated with [$^3$H]inositol (2 mM) and [$^{14}$C]choline (2 mM) in HEPES buffer. [$^3$H]Phosphatidylinositol ([$^3$H]PI) and [$^{14}$C]phosphatidylcholine ([$^{14}$C]PC) are represented by (●, ○) and (▲, △) in vehicle and ethanol, respectively. Data are the means ± SE from 5 embryos; and (★) and (★★) significantly different from vehicle, $P < 0.05$ and $P < 0.01$, respectively.

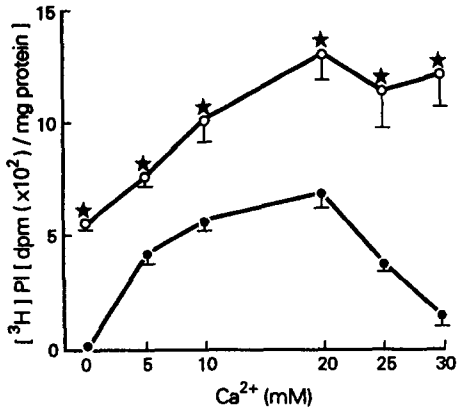


Fig. 2. Effect of *in vivo* administration of ethanol on sodium- and calcium-dependent [$^3$H]inositol incorporation in brain synaptosomal phosphatidylinositol ([$^3$H]PI). The isolated synaptosomes were incubated in both normal HEPES buffer and a sodium-free medium. Incorporation values obtained with the latter medium were an indication of sodium-independent incorporation and were subtracted in all cases from the values obtained utilizing the former medium. Data are the means ± SE from 5 embryos. Vehicle and ethanol are represented by (●) and (○), respectively; and (★) significantly different from vehicle, $P < 0.01$.

of the supernatant (1.0 mL) were transferred to 10 mL of ACS II for the determination of $^{45}Ca^{2+}$ released into the incubation medium from synaptosomes.

Statistical analysis was performed using Student's *t*-test (two-tailed).

## RESULTS

### *Effect of ethanol on incorporation of* [$^3$H]*inositol and* [$^{14}$C]*choline*

Incorporation of both [$^3$H]inositol and [$^{14}$C]choline as greater in the ethanol group than in the vehicle group from 0–30 min (Fig. 1). Incorporation of [$^3$H]inositol into PI was stimulated by 5–20 mM of $Ca^{2+}$ in both vehicle and ethanol groups. In the absence of added $Ca^{2+}$, ethanol significantly stimulated the basal rate of [$^3$H]inositol incorporation into synaptosomal PI (Fig. 2). In addition to its effects on the basal rate of incorporation, ethanol produced significant increases in the $Ca^{2+}$-stimulated incorporation of [$^3$H]inositol into PI at $Ca^{2+}$ concentrations of 5–30 mM with 125 mM of sodium ($Na^+$). Incorporation of [$^{14}$C]choline into PC was also stimulated by 5–20 mM of $Ca^{2+}$ in both vehicle and ethanol groups. In the ethanol group $Na^+$- and $Ca^{2+}$-dependent incorporation via base exchange of [$^{14}$C]choline into PC was significantly higher than that in the control group (Fig. 3). Significant differences were observed at all $Ca^{2+}$ concentrations.

The incorporation via base exchange reaction required normal $Na^+$ and $Ca^{2+}$ concentrations in the incubation medium.

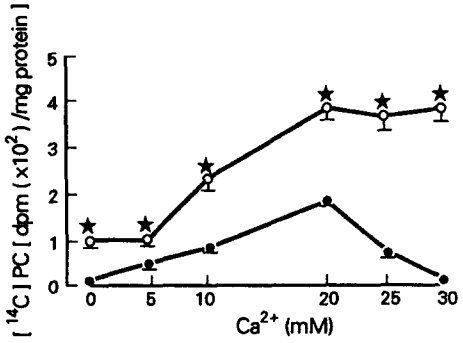


Fig. 3. Effect of *in vivo* administration of ethanol on sodium- and calcium-dependent [$^{14}$C]choline incorporation in brain synaptosomal phosphatidylcholine ([$^{14}$C]PC). The experiments were done as described in Fig. 2. Data are the means ± SE from 5 embryos. Vehicle and ethanol groups are represented by (●) and (○), respectively; and (★) significantly different from vehicle, $P < 0.01$.

[$^3$H]Inositol and [$^{14}$C]choline incorporation were also studied in brain and heart synaptosomes after ethanol administration on the third day of embryogenesis. As shown in Table 1, ethanol increased to $Ca^{2+}$- and $Na^+$-stimulated exchange of brain [$^3$H]inositol to 144.6% of the control value, and decreased the basal rate of [$^3$H]inositol incorporation into heart synaptosomal PI to 78% of the control value. [$^{14}$C]Choline exchange increased to 119.8% of the control value in the brain, and the basal rate of [$^{14}$C]choline incorporation into heart synaptosomal PC decreased to 73.6% of control.

Table 1. Effect of *in vivo* administration of ethanol on [$^3$H]inositol or [$^{14}$C]choline incorporation into embryo brain and heart phosphatidylinositol (PI) and phosphatidylcholine (PC)

| | | dpm/$\mu$g $P_i$ | |
|---|---|---|---|
| | | Brain | Heart |
| [$^3$H]PI | Vehicle | 100.00 ± 2.53 | 100.00 ± 7.37 |
| | Ethanol | 144.60 ± 1.72† | 78.05 ± 3.52† |
| [$^{14}$C]PC | Vehicle | 100.00 ± 1.88 | 100.00 ± 4.74 |
| | Ethanol | 119.82 ± 7.42† | 73.60 ± 4.68* |

Ethanol was injected to eggs on the third day of embryogenesis. Brain and heart were taken from the 20-day-old embryos.

Values are means ± SE from 6 experiments, and are expressed as percentage of vehicle values of dpm/$\mu$g $P_i$ (inorganic P: phospholipid-P).

* $P < 0.05$ and † $P < 0.02$.

Table 2. Effect of *in vivo* administration of ethanol on $^{45}Ca^{2+}$ uptake into embryo brain (a) and heart (b) synaptosomes

| | $Ca^{2+}$ (M) | dpm/mg protein × $10^3$ Vehicle | Ethanol |
|---|---|---|---|
| (a) | $10^{-7}$ | 107.6 ± 2.7 | 80.3 ± 3.8* |
| | $10^{-6}$ | 112.4 ± 2.4 | 78.9 ± 2.6* |
| | $10^{-4}$ | 41.2 ± 1.9 | 21.8 ± 0.7* |
| (b) | $10^{-7}$ | 89.4 ± 4.6 | 217.3 ± 2.1* |
| | $10^{-6}$ | 68.3 ± 3.4 | 193.5 ± 11.9* |
| | $10^{-4}$ | 41.7 ± 4.8 | 95.2 ± 6.9* |

Ethanol was injected to eggs on the third day of embryogenesis. Brain and heart were taken from 20-day-old embryos.

Values are mean ± SE from 6 experiments.

* Significantly different from vehicle $P < 0.001$.

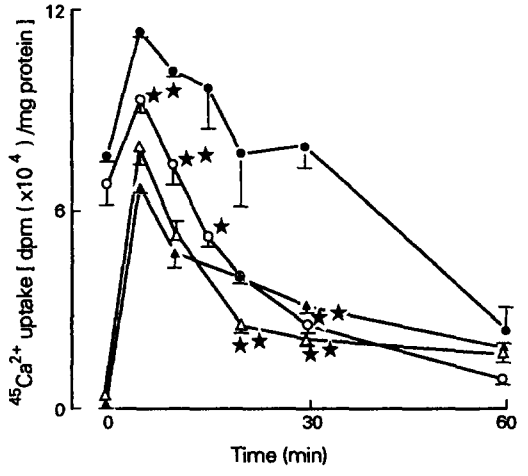


Fig. 4. Time course of changes in $^{45}Ca^{2+}$ uptake in brain and heart synaptosomes. Synaptosomes were isolated from treated or untreated embryos. Data are means of 3 embryos. Key: (●) brain-vehicle; (○) brain-ethanol; (▲) heart vehicle; (△) heart-ethanol; (★) and (★★) significantly different from vehicle, $P < 0.05$ and $P < 0.01$, respectively.

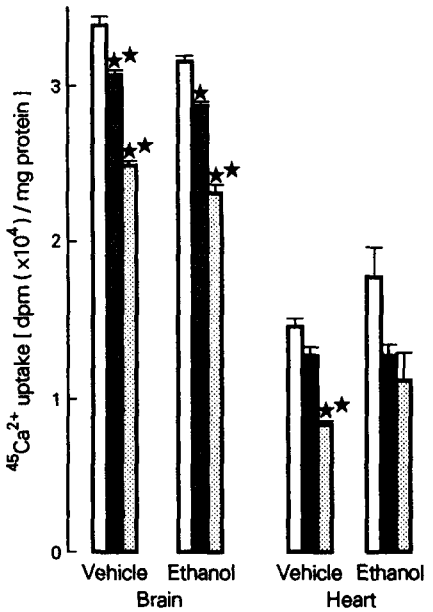


Fig. 5. Effect of *in vitro* addition of ethanol on $^{45}Ca^{2+}$ uptake in brain and heart synaptosomes from control and ethanol-treated embryos. $^{45}Ca^{2+}$ uptake was determined as described in the text. Data are means ± SE of 6 determinations. Key: (□) 0; (▨) 100; (▩) 400 mM ethanol; (★) and (★★) significantly different from control, $P < 0.05$ and $P < 0.01$, respectively.

*Effect of ethanol on* $[Ca^{2+}]_i$

In brains which had not been treated with ethanol $[Ca^{2+}]_i$ was 593 nM, while in those which had been treated it was 806 nM. On the other hand, the concentrations of $[Ca^{2+}]_i$ in the heart with and without ethanol treatment were 505 and 648 nM, respectively. Ethanol treatment significantly increased $[Ca^{2+}]_i$ in the brain, but it significantly decreased in the heart. In the *in-vitro* study, neither heart nor brain $[Ca^{2+}]_i$ was affected by addition of 1–5% ethanol (data not shown).

*Effect of ethanol on* $^{45}Ca^{2+}$ *uptake*

Figure 4 shows the time course of $^{45}Ca^{2+}$ uptake in brain and heart for 60 min. Uptake in brains from the ethanol group was less than in the control group. Uptake in hearts from the ethanol group was greater than in the control group, but only for the first 10 min. In brains from ethanol-treated animals, $^{45}Ca^{2+}$ uptake was 25.4, 29.8 and 47.0% less than in control animals' brains, in the presence of $10^{-7}$, $10^{-6}$ and $10^{-4}$ M extrasynaptosomal $Ca^{2+}$, respectively. On the other hand, in ethanol-treated hearts $^{45}Ca^{2+}$ uptake was 143.1, 183.5 and 128.5% greater than the control values in the presence of $10^{-7}$, $10^{-6}$ and $10^{-4}$ M extrasynaptosomal $Ca^{2+}$, respectively (Table 2). In the *in-vitro* study addition of ethanol decreased $^{45}Ca^{2+}$ uptake in both organs under all conditions (Fig. 5).

Incubation with ethanol did not stimulate release of $^{45}Ca^{2+}$ from synaptosomes of either organ in either the control or the ethanol-treated group (data not shown).

## DISCUSSION

Ethanol enters the hydrophobic regions of the cell membrane causing an increase in membrane fluidity [21]. The change in fluidity may result from a disturbance of the dynamic equilibrium of membrane phospholipids. Many membrane-bound enzymes are affected by their fluid environment and membrane fluidity is positively correlated with membrane transport activity [22]. This effect of ethanol on membranes may contribute to the alteration of calcium mobilization.

$\alpha_1$-Adrenoceptor activation has also been shown to be associated with increased PI metabolism (turnover) in numerous tissues such as mammalian brain [23], liver [24] and vascular smooth muscle [25, 26]. However, it is still uncertain whether PI turnover is involved directly in mobilizing intracellular $Ca^{2+}$ stores, or in $Ca^{2+}$ gating, or in both processes. PI and PC turnover, as measured by release of inositol-, choline-phosphate and DAG, suggests that PI may not be the only precursor of DAG [27–29]. The evidence presented here indicates that ethanol affects PI and PC turnover in different ways depending on the organ. In addition, alterations in PI- and PC-turnover, $[Ca^{2+}]_i$ and $Ca^{2+}$ uptake were noted in brain and heart after prenatal ethanol exposure. However, the mechanism and molecular basis of membrane tolerance of ethanol remains obscure. The assays of $[Ca^{2+}]_i$ and of $^{45}Ca^{2+}$ fluxes differ considerably from a methodological point of view. $[Ca^{2+}]_i$ measurement is independent of synaptosomal number whereas $^{45}Ca^{2+}$ fluxes diminish if some synaptosomes are disrupted. The data indicating reduced PI- and PC-turnover and $^{45}Ca^{2+}$ uptake *in vitro* must be interpreted with caution, as $Ca^{2+}$ entry blockage and synaptosomal lysis can give identical results. In addition, Taraschi *et al.* [6] have reported that PI isolated from ethanol-fed rats caused a small decrease in the level of arachidonic acid and a slight increase in the quantity of oleic acid. These results differ from those reported by Cunningham *et al.* [30], but the reasons for this discrepancy are not clear. Chronic ethanol treatment may also change the phospholipid molecular species, although evidence for such alterations have not yet been sought. It has recently been reported that minute changes in the molecular species of erythrocyte PC alter the physical properties of membranes [31]. Also, Hoffman *et al.* [32] have reported that differential responses to ethanol of $\beta$-adrenergic receptors in heart and brain exemplify the specificity of ethanol's actions on various organ systems.

It is concluded that alteration in PI- and PC-turnover and $Ca^{2+}$ mobilization may also contribute to membrane tolerance in the differential responses to ethanol in chick embryo brain and heart.

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