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Comparison of Effects of Ethanol on Platelet Function and Synaptic Transmission

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FENN, G. C., M. A. LYNCH, P. T. NHAMBURO, L. CABEROS AND J. M. LITTLETON. *Comparison of effects of ethanol on platelet function and synaptic transmission.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 37–43, 1983.—Ethanol, at concentrations tolerated by man, is generally inhibitory to platelet function *in vitro*, producing significant inhibition of aggregation in response to most chemical aggregating agents. The calcium ionophore, A23187, is the agent which is most inhibited by ethanol, whereas aggregation induced by arachidonic acid is not inhibited even by concentrations of ethanol far in excess of the lethal range. This spectrum of inhibition found suggests that ethanol inhibits the platelet release reaction by a mechanism involving inhibition of Ca^{2+}-activated phospholipase A_2. These effects can be observed in superfused human platelets as well as in those suspended in buffer or in plasma. In superfused rat brain slices however, ethanol does not inhibit the A23187-induced release of radiolabelled neurotransmitter, although it can be shown to inhibit Ca^{2+}-activated phospholipase A_2 activity in rat synaptosomal preparations. It is concluded that, although there are many similarities between the effects of ethanol on the platelet and the synapse there may be differences between the way in which ethanol modifies release of intracellular contents in the two situations.

Ethanol Platelet aggregation Ca^{2+} ionophore A23187 Arachidonic acid Neurotransmitter release Phospholipase

MANY workers have used the blood platelet as a model system for the mammalian neurone [13]. One reason for this use is the fact that the platelet is an example of an easily obtainable human cell which performs many of the functions of the neurone. For example it is capable of taking up exogenous amines and storing them in intracellular vesicles [13], and it will release the contents of these vesicles after stimulation by certain agents as a consequence of an increase in cytosolic Ca^{2+} concentration [9]. In addition, the platelet membrane contains receptors for a wide variety of compounds with presumed neurotransmitter activity in mammalian brain. Thus, mammalian platelets of various species contain receptors for 5-hydroxytryptamine, catecholamines, vasopressin, ADP, and acetylcholine as well as receptors for those compounds present in plasma and in the vessel wall such as thrombin and collagen, which also modify the function of platelets [3,12]. Given the large amount of literature which surrounds the interaction of alcohols and anaesthetics with cells such as red blood cells [14], and which attempts to extrapolate this information to the mechanism of action of these compounds on neurones, it seems odd that there is relatively little published work on the effects of alcohols and anaesthetics on blood platelets.

Such work as has been published suggests that alcohols have an inhibitory effect on the amine uptake process [10], and that their effect on the release reaction of platelets varies considerably with the aggregating agent used to induce release [4]. Similar results have been reported with some anaesthetics [2]. We have recently described experiments which show that the inhibitory effect of ethanol on the platelet release reaction depends in part on the membrane lipid composition of the platelet, and that the effects of ethanol on platelet function are in many ways compatible with a membrane "fluidising" effect of the drug [5]. If the assumption is made that ethanol acts within the membrane lipid bilayer to disrupt platelet function, then it seems likely that the key enzymes which are affected are those which have as their substrates the lipids of the membrane, and which are themselves likely to be located within the membrane. Figure 2 shows a simplified version of the way in which these enzymes are thought to act on platelet membrane lipids to initiate platelet aggregation and release. Since many of these reactions also occur in synaptic membranes and are associated with transmitter release, it is of interest to compare the effects of ethanol on the secretion of platelet vesicle contents and on the secretion of neurotransmitters by mammalian neurones. In both instances effects of ethanol on membrane lipid metabolizing enzymes have been, or are in the process of being, investigated by us.

This paper extends our previous work on human platelets

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[4, 5, 6, 11] to consider the effect of different alcohols on secretion, and follows the implications of this work for synaptic transmission. In particular the role of calcium entry and the role of sensitivity to Ca^{2+} of the process of vesicular release are considered in platelet and synapse.

METHOD

Platelet Aggregation

Preparation of platelet rich plasma (PRP). Blood was taken from healthy human volunteers via a butterfly needle into a plastic syringe containing 1/10 volume standard acid citrate:dextrose anticoagulant. Whole blood was centrifuged at 180×G for 20 min, then PRP was removed and further centrifuged at 200×G for 5 min to remove contaminating red cells.

Preparation of gel-filtered platelets (GFP). PRP was centrifuged at 1000×G for 20 min at room temperature onto a cushion of 40% (w/v) bovine serum albumin. The platelet pellet was passed through an 80 cm Sepharose CL2B column in the presence of apyrase. The elution buffer was calcium-free Tyrode solution (pH 7.3).

Platelet aggregation. Aggregation was studied turbidimetrically with continuous reading of light transmission, using 500 μl samples (at 37°C and stirred at 1000 rpm) in either a modified Born RCS 2-channel monitor or a Payton Dual Channel Aggregation Module.

Platelet release reaction (luciferin luciferase). The platelet release reaction was induced by addition of the Ca^{2+} ionophore A23187 to unstirred GFP in a cuvette in a spectrophotometer. After a stimulus, platelets release their contents, including ATP, which acts as a substrate for the luciferin-luciferase system, resulting in an increase in luminescence which is quantitated by a light detector.

Superfusion of Platelets

PRP was incubated for 60 min at room temperature with ^{3}H-5HT (25 μCi/20 ml PRP) and after centrifugation onto bovine serum albumin at 1000+G (25 min) platelets were removed by aspiration. Platelets were then immobilised on Millipore filters (0.8 μm pore diameter) and superfused with Ca^{2+}/Mg^{2+}-free Krebs solution containing 1 mM EGTA (pH 7.35, 290±10 m OSM).

After 30 min of "free" superfusion (during which no collection was made) fraction collection was initiated for a period of 110 min during which the platelets were stimulated twice (S_1, S_2) for 7 min with Ca^{2+} 0.5 mM and A23187 2.5 μm.

In some experiments 50 mM Etoh was added to the perfusate during S_1. The effect of omitting Ca^{2+} from the perfusage in S_1 and S_2 on A23187 induced ^{3}H release was also studied (see Fig. 5).

Fractional release of ^{3}H-5HT was estimated in each fraction collected (dpm remaining on the filter paper at the end of superfusion was measured and the amount present before collection of each fraction was calculated from this).

Brain Slice Superfusion

Rats (Sprague Dawley males, 200–250 g) were killed by stunning and decapitation. The brain was rapidly removed and the striatum dissected on ice. Striatal prisms (0.2×0.2×0.2 mm) were prepared by using a McIllwain tissue chopper. The prisms were incubated at 37°C in 10 ml Krebs solution containing 50 μl ^{3}H-dopamine (^{3}H-DA) for 1 hr and were then mounted on filter paper and placed in a tissue holder. Prisms were then superfused with oxygenated Krebs solution, at 37°C and 1 hr later collection of samples commenced. In control experiments Krebs solution contained 12 μM A23187 during two periods of stimulation (S_1 and S_2), at 25 minutes and at 80 minutes (see Fig. 6). To examine the effect of alcohol on ^{3}H-DA release, 50 mM ethanol was added to Krebs solution for 45 minutes prior to S_2 and to the Krebs-A23187 solution during S_2. Fractional release of ^{3}H-DA was estimated in each fraction collected.

Synaptosomal Phospholipase A2 Activity

Preparation of synaptosomes. Male Sprague-Dawley rats (200–250 g; Charles River U.K.) were killed by decapitation, the whole brain dissected in the cold and synaptosomes prepared by sucrose density gradient centrifugation.

Preparation of emulsions of phospholipids. Appropriate amounts of 1-acyl-2[^{3}H]oleoyl-phosphatidylcholine were added to a sonicating vessel. The lipid was then dried in N_2 gas at room temperature. The dried sample was redissolved in the appropriate volume of Tris-HCl/NaCl buffer pH 9.0 and the mixture was then sonicated (12,000 Hz) in a vessel surrounded by ice for 2 periods of 1 min, with a 30 sec interval between each period. The sonicated lipid was used within 2 hours of preparation.

Conditions of incubations. Freshly prepared synaptosomes were incubated in 100 mM Tris-HCl, 0.8% NaCl buffer pH 9.0 containing 2 mM $CaCl_2$, sonicated lipid and 50 mM ethanol (or distilled water) for the appropriate time at 37°C. The reaction was terminated by addition of 3 mls of ice-cold chloroform:methanol:HCl (2:1:0.02, v/v/v) and mixed vigorously for 15 seconds before placing on ice. To remove non-lipid substances, 0.2 volume of distilled water was added, mixed thoroughly and centrifuged at 3,000 rpm for 10 min. The aqueous layer was removed and re-extracted in 2 mls of the organic solvent. The process was repeated.

The combined extracts in organic solvent were dried under N_2 gas. Dried lipid was then resuspended in 0.15 ml chloroform. Aliquots of lipid solution (100 μl) were then chromatographed in a band 3 cm wide on silica-gel glass plate and developed in a solvent system of chloroform: methanol:water and concentrated ammonia (65:25:4:0.04, v/v/v/v) to a distance of 17 cm from the origin. Components were detected in iodine vapour, positions marked and after evaporation parts of chromatograms containing oleic acid, phosphatidylcholine and lysophosphatidylcholine were scraped and transferred into vials. Ten ml of scintillant (PPO and 2-ethoxyethanol) were then added and samples were mixed thoroughly before scintillation counting.

Materials

All chemicals were obtained from Sigma, and radiochemicals from Amersham (U.K.).

RESULTS

Platelet Aggregation

Ethanol inhibits the aggregation of human platelets (either platelet-rich plasma or gel-filtered platelets) when aggregation is caused by any agent tested except exogenous arachidonic acid. At even molar concentrations the effect of ethanol on arachidonic acid-induced aggregation is a small but significant enhancement of the rate of aggregation (Fig. 1A). Of the "physiological" aggregating agents tested, colla-

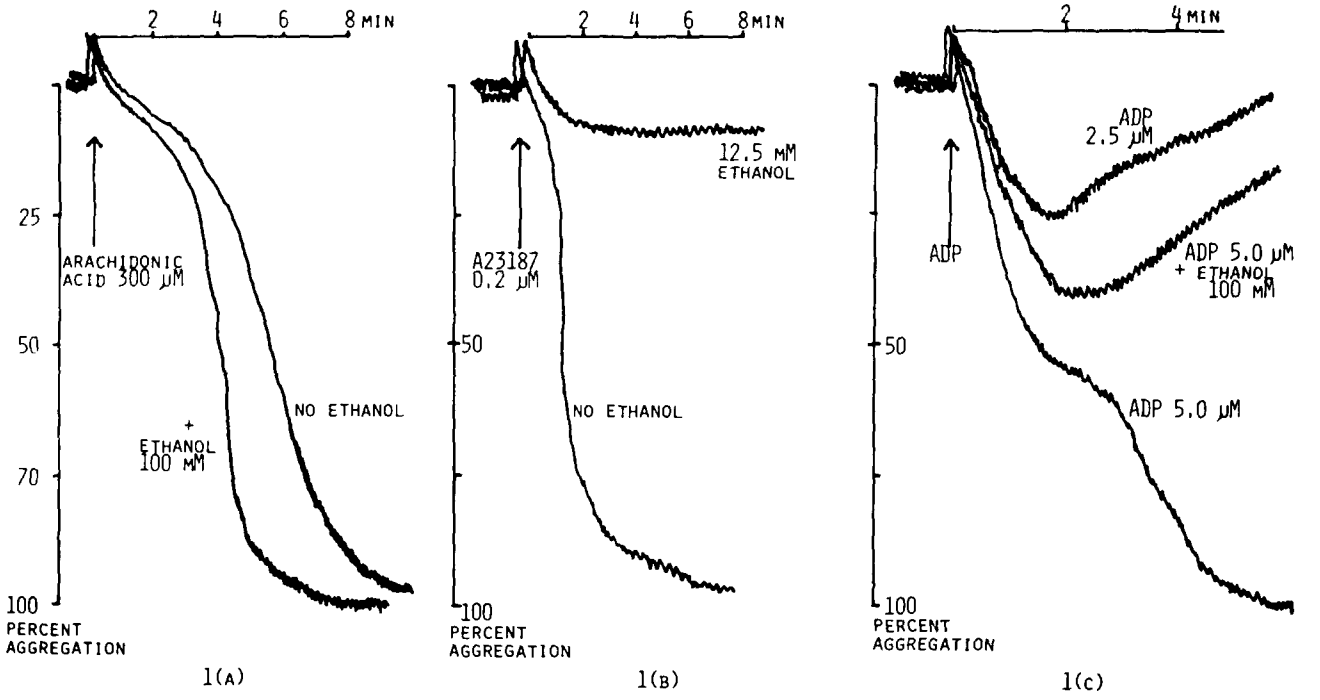

FIG. 1. Effect of 2 min preincubation with ethanol on gel-filtered platelet aggregation induced by different agents. (A) Potentiation of arachidonic acid-induced aggregation. When compared in a paired Student's *t* test ethanol significantly reduced the time to reach half maximal aggregation ($p<0.01$). (B) Inhibition of A23187-induced aggregation. (C) Inhibition of secondary wave of ADP-induced aggregation.

gen and thrombin were most inhibited by ethanol, with IC_{50} values for ethanol of 25–50 mM being obtained in some individuals. However, the agent which was by far the most sensitive to inhibition by ethanol was the calcium ionophore A23187. Figure 1B shows representative traces in which almost complete inhibition of A23187-induced aggregation was produced by 12.5 mM ethanol in platelets from one individual. Platelets from many other individuals were similarly sensitive. Those aggregating agents most sensitive to ethanol, i.e., collagen, thrombin and A23187, have in common the property of causing aggregation virtually simultaneously with the release reaction (which releases further pro-aggregatory factors) and all probably act by a mechanism involving an increase in free intracellular calcium [15]. In all instances, where aggregation was associated with a biphasic response (ADP-, adrenaline-, 5-hydroxytryptamine-induced aggregation) ethanol was always a more effective inhibitor of the second phase, i.e., that associated with release (Fig. 1C).

When different alcohols were investigated for their inhibitory effect on ADP- or collagen-induced aggregation there was a loose relationship between inhibitory potency and lipid solubility. However, the shorter chain alcohols, and particularly ethanol, were more potent than their lipid solubility would indicate if this were the only factor determining platelet inhibitory potency (Fig. 3).

Platelet Release Reaction

The above results suggest strongly that ethanol has its major effect by inhibiting the platelet release reaction and that release induced by A23187 was most sensitive to inhibition by ethanol. We therefore attempted to investigate release of platelet contents by A23187 in conditions where aggregation itself is a less important factor in initiating release than it is in a stirred (aggregometer) cuvette. Release induced in gel-filtered platelets by A23187 in a spectrophotometer cuvette (unstirred) is shown in Fig. 4. The release of platelet contents (including ATP) was measured by the increase in luminescence of luciferin-luciferase. Ethanol inhibited release in a dose-related way.

Platelets preloaded with ^{3}H-5HT and then superfused with physiological fluid containing A23187, released ^{3}H into the superfusate. A second period of superfusion (S_2) with A23187 always caused a smaller fractional release of ^{3}H into the superfusate than did the first (S^1). The release of ^{3}H from platelets by A23187 was dependent on the presence of Ca^{2+} in the superfusate. The maximum rate of release of ^{3}H during the S_1 period was highly correlated (correlation coefficient = 0.96) with the calculated dpm present in platelets on the filter at the beginning of the experiment (this is an index of the number of viable platelets present on the filter paper). This relationship suggests that platelet:platelet contact enhances the release of platelet contents by A23187 (see Discussion).

When ethanol 50 mM was present with A23187 during S_1 in experiments where large numbers of viable platelets were present, the rate of ^{3}H release was inhibited. Where smaller numbers of viable platelets were seen the effect was less clear-cut. The presence of ethanol during S_1 always resulted in a greater fractional release of ^{3}H during the second challenge with A23187 (S_2) indicating a greater fraction of releasable ^{3}H remaining in preparations where ethanol was included (Fig. 5). The results indicate that ethanol reduces A23187-induced release of platelet contents particularly when platelets are in close contact with each other.

Brain Slice Superfusion

In contrast to the inhibitory effects of ethanol on release

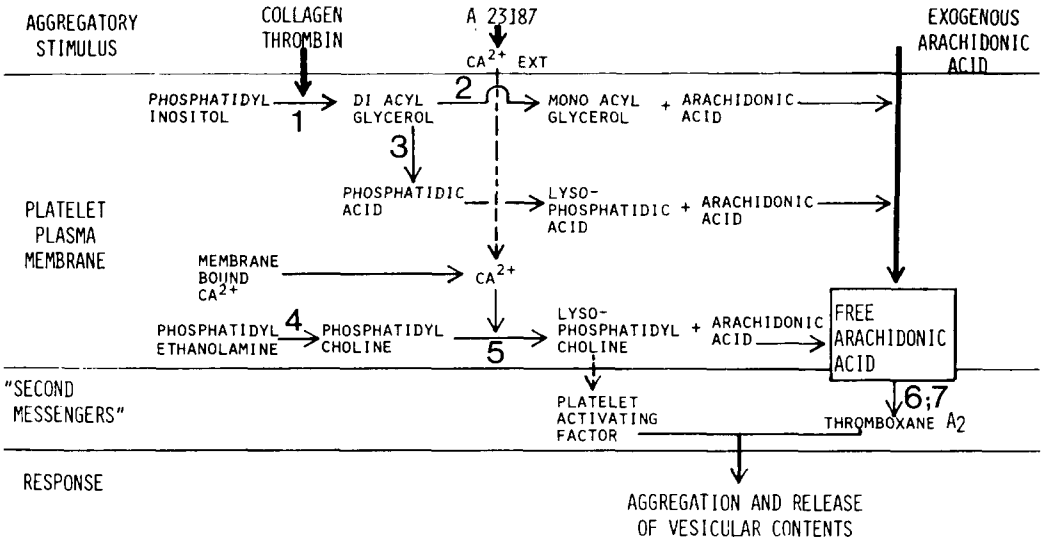

FIG. 2. Postulated roles of membrane phospholipids and enzymes in platelet aggregation and release of vesicular contents. Enzymes indicated with numerals are (1) Phospholipase C; (2) Diacylglycerol (D.A.G.) Lipase; (3) D.A.G. Kinase; (4) Transmethylation enzymes; (5) Phospholipase A_2; (6) Cyclooxygenase; (7) Thromboxane Synthetase.

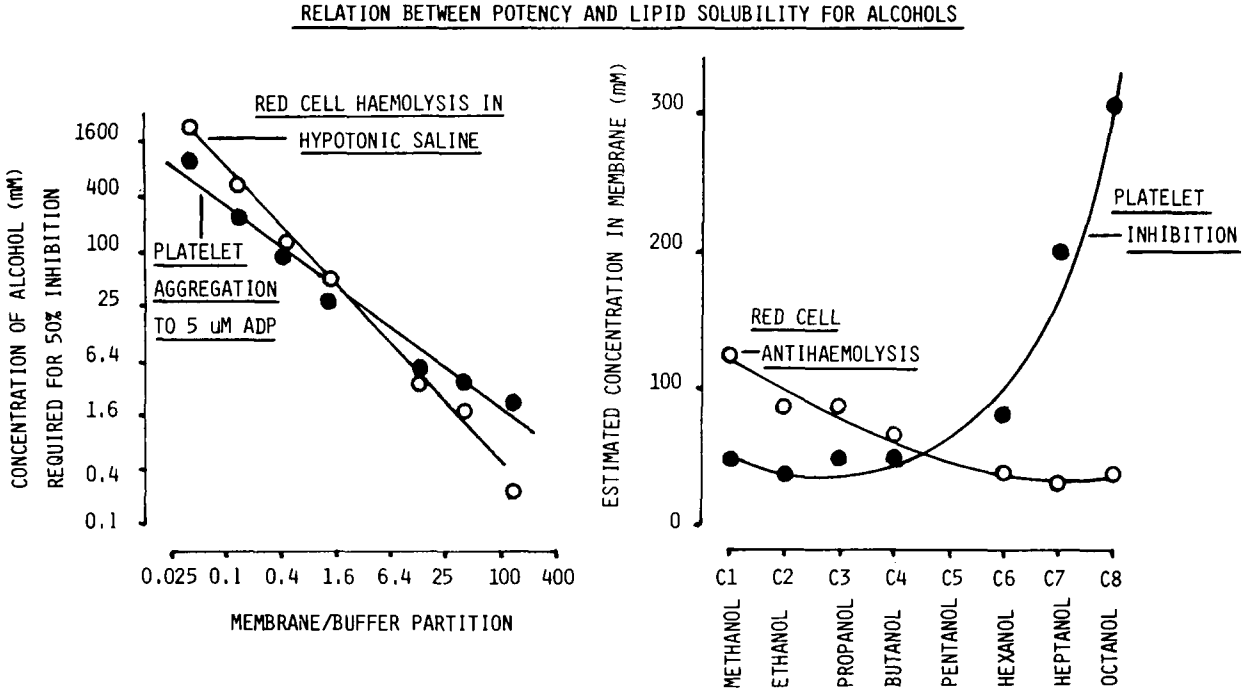

FIG. 3. Relationship between inhibitory potency of alcohols of different chain lengths (on ADP-induced aggregation) and their membrane lipid solubility. For comparison shows the red cell antihaemolysis effect of the different alcohols.

of platelet contents in response to A23187, the release of ^{3}H-dopamine from rat corpus striatum slices produced by 12 μM A23187 was enhanced by the presence of ethanol, 50 mM, in the superfusing fluid (Fig. 6).

Synaptosomal Phopholipase A2 Activity

The effect of ethanol on synaptosomal phospholipase A_2 activity is shown in Fig. 7. As can be seen ethanol inhibits the activity of this enzyme in brain at concentrations achieved *in vivo*.

DISCUSSION

The results obtained using human platelets are entirely compatible with the hypothesis that ethanol inhibits platelet aggregation by reducing the platelet release reaction. Based on the scheme outlined in Fig. 2, this action seems likely to

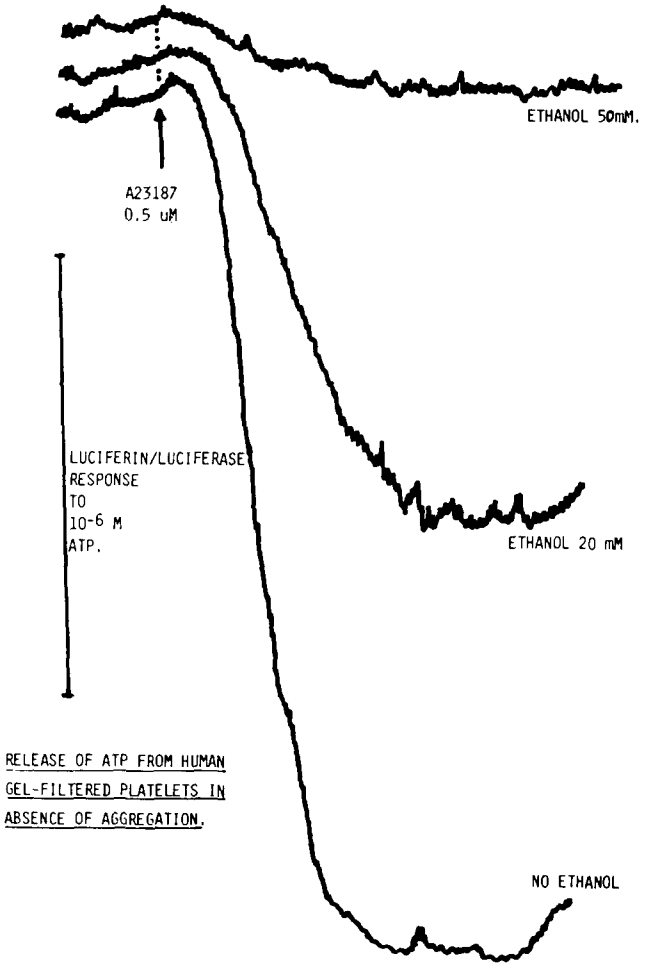

FIG. 4. Effect of 2 min preincubation with ethanol (20 mM and 50 mM) on the release reaction induced by calcium ionophore A23187, measured by the luciferin-luciferase response.

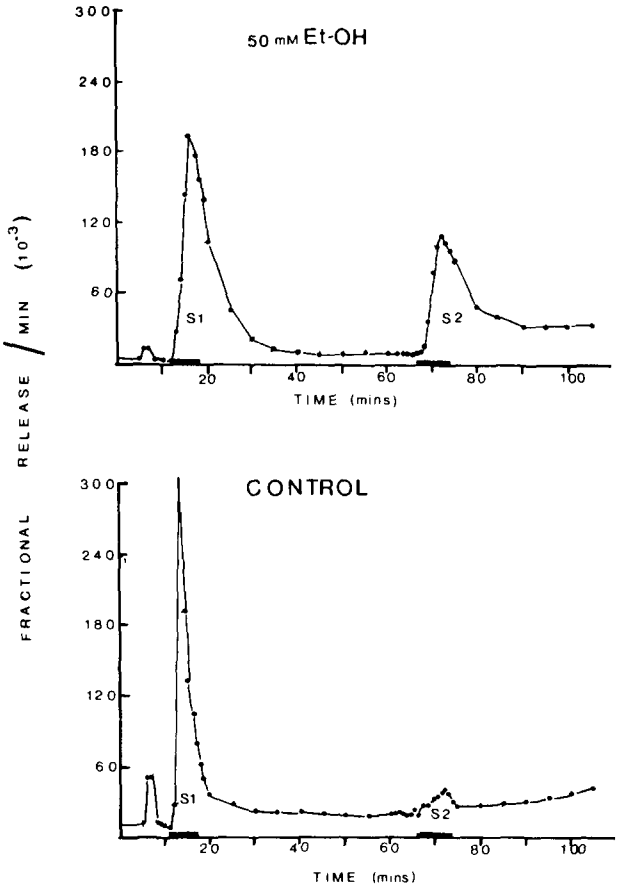

FIG. 5. Effect of in vitro ethanol on the release of ^{3}H-5HT from human platelets. Platelets were superfused with Ca^{2+}/Mg^{2+}-free Krebs solution containing 1 mM EGTA. The release of ^{3}H-5HT was stimulated for 7 min with Krebs solution containing Ca^{2+} (0.5 mM) and A23187 (2.5 μM). Ethanol (50 mM) was included in the superfusate during S_1.

be due to inhibition of the activation by Ca^{2+} of phospholipase A_2 since A23187-induced release as well as release induced by collagen and thrombin, are very much affected by ethanol, whereas arachidonic acid-induced release is not inhibited by ethanol. The potency of alcohols of differing chain length (as well as our previously reported experiments on incorporation of different lipids into the platelet membrane [4,5]) suggest that these effects may be similar to a membrane "fluidisation" in which less-saturated acyl chains are found in membrane phospholipids. Extrapolating from these platelet results to the neurone, we expected to find that synaptic phospholipase A_2 would be inhibited by ethanol at low concentrations, and also that release of neurotransmitter induced by A23187 would be inhibited by ethanol. This would have strongly implicated an interaction with depolarisation-induced increased Ca^{2+} levels as being an important site for the action of ethanol on synaptic transmission. However, although we obtained significant inhibition of Ca^{2+}-activated phospholipase A_2 by ethanol in synaptosomal preparations, we obtained no inhibition (but rather enhancement) of A23187-induced release of neurotransmitter in superfused rat corpus striatum slices.

The reason for this discrepancy is not immediately apparent, but it may reside in differences between the stimuli for the platelet release reaction and the neuronal release of transmitter even when the same trigger, A23187, is used. Thus, in platelets it has been suggested that the A23187-induced release of contents is dependent on close platelet-platelet contact (i.e., aggregation) and on the subsequent metabolism of products of phospholipid breakdown by phospholipase A_2 [7]. We cannot exclude platelet-platelet contact in our systems of measuring release and indeed some of the superfusion studies enhance this contact. The inhibitory effect of ethanol on release could therefore be due to an effect on the mechanism by which platelet-platelet contact initiates the release reaction. Since such a mechanism is unlikely to play any part in neurotransmitter release by A23187 it may not be surprising that ethanol affected neurotransmitter release differently.

The situation may be analogous to the discrepancy between the effects of ethanol on transmitter release induced in brain slices by brief pulses of electrical depolarisation (inhibition [1]), or by relatively prolonged periods of high $[K^+]$ depolarisation (no effect [8]). In both instances, release of transmitter is probably due to opening of voltage-dependent Ca^{2+} channels and the subsequent increase in intracellular Ca^{2+} levels. The discrepancy can be explained if ethanol decreases the open time of Ca^{2+} channels exposed to brief

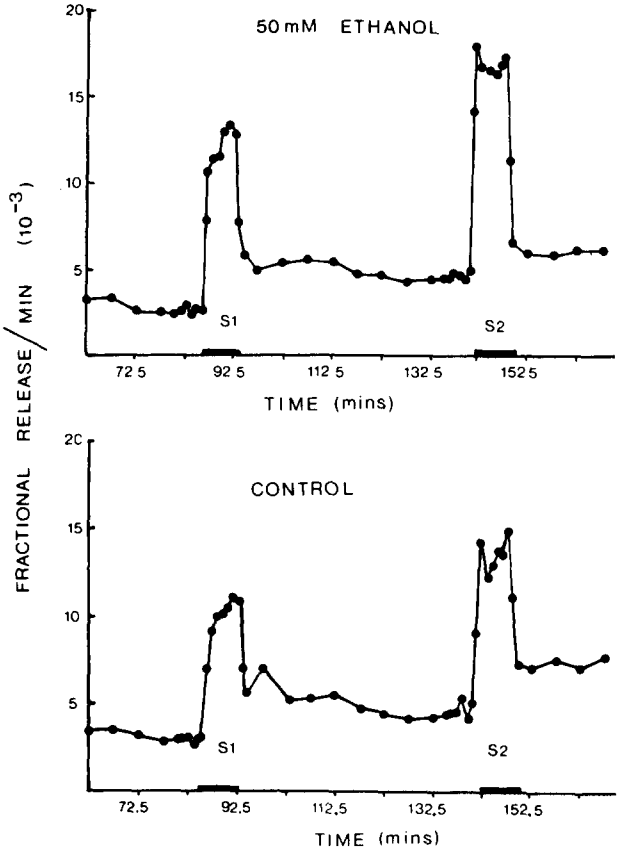

FIG. 6. Fractional release (FR) of ^{3}H-DA ($\times 10^{-3}$) from rat striatal slices induced by 12 μM A23187. Slices were superfused with Krebs solution throughout the experiment with the addition of 12 μM A23187 during S_1 and S_2. In the ethanol experiment, 50 mM ethanol was included in the superfusing Krebs solution for 45 minutes prior to S_2 and was present with A23187 during S_2. Mean FR ($\pm$S.E.M.) in S_1 and S_2 was calculated by subtracting basal release from stimulated release. Mean FR in control S_1 was 6.98 ($\pm$0.27) and S_2 was slightly increased to 8.41 ($\pm$0.71). In the ethanol experiment FR in S_1 was 8.64 ($\pm$0.68) which was significantly increased to 11.85 ($\pm$0.43) in S_2 ($p<0.01$ by Student's t-test).

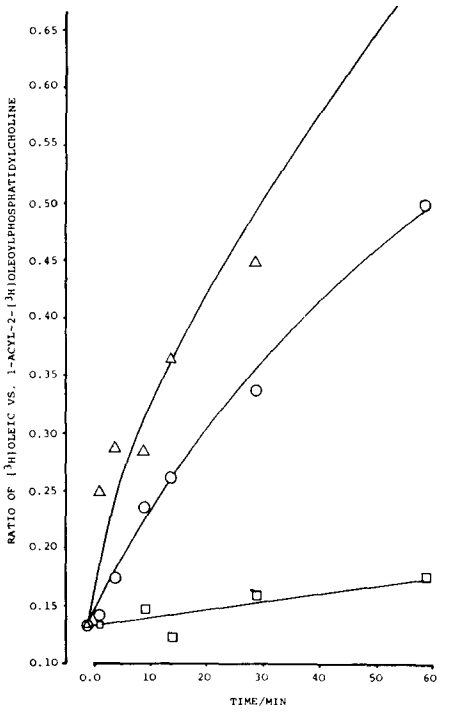

FIG. 7. Time course of hydrolysis of 1-acyl-2[^{3}H]oleoyl-phosphatidylcholine by rat brain synaptosomes in the presence (○) and absence (△) of 50 mM ethanol. Incubation conditions are as described in the text. (□) represents spontaneous hydrolysis of label at 37°C.

voltage changes. It may be that, similarly, in the platelet ethanol decreases the Ca^{2+} permeability of platelets exposed to brief mechanical stimuli associated with platelet-platelet contact. Whatever explanation is correct there are clearly similarities and differences in the effects of ethanolon platelet and synaptic function.

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