of rat brain. These results do not support the hypothesis that the TRH antagonism of drug-induced narcosis is mediated by modulating the release of ACh from cholinergic neurones which form synapses in the nucleus accumbens or septum.

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Precursor- and pool-dependent differential effects of ethanol on human platelet prostanoid synthesis

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Ethanol-induced changes in prostanoid synthesis occur both *in vitro* [1–4] and *in vivo* [5] and in a variety of tissues and species [6, 7]. Effects of ethanol on prostaglandin-stimulated adenylate cyclase activity are reported as well [1, 8, 9]. Chronic ethanol administration also inhibits the desaturases that convert *cis*-linoleic acid (cLA) to gamma-linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA) to arachidonic acid (AA) in liver and brain [10, 11]. Many of these effects occur within physiologically relevant concentration ranges of ethanol.

These biochemical effects of ethanol on essential fatty acid (EFA) metabolism and prostanoid synthesis may underlie diverse *in vivo* effects of ethanol. Evidence supporting this is that in rodents manipulation of EFA metabolism and prostanoid synthesis with non-steroidal anti-inflammatory drug, EFA-deficient diets or with prostaglandin (PG) precursor EFA treatment alters behavioral, physiologic and pathologic effects of ethanol, including: sedation [12, 13], hyperactivity [14], hypothermia [15], withdrawal behavior [1, 13, 16] and the development of fatty liver measured by the accumulation of hepatic triglycerides [17, 18].

Further, treatment of human alcoholics with EFAs (10% GLA, 72% cLA) may reduce alcohol craving, improve liver function, and enhance impaired cognitive function [19].

Given the vast amounts of EFAs esterified to membrane phospholipids (much of which are available for prostaglandin synthesis), it is not clear how to account for the potent effects of dietary EFAs on the behavioral response to ethanol. Based on analysis of eicosanoid metabolism in rabbits fed an EFA-deficient diet, Crawford [20] has proposed two physiologically active pools for prostaglandin synthesis: (1) a "membrane" pool of esterified EFAs (not readily affected by diet) released upon phospholipase activation, and (2) a "metabolic" precursor pool of free EFAs (sensitive to dietary changes) which may play an important role in maintaining prostanoid levels under non-stimulated conditions. We have studied this concept in pulse-labeled platelets (in which nearly all labeled precursor EFAs are esterified to phospholipids) and in platelets incubated briefly with free (non-esterified) EFAs. We now report data which (1) may help to clarify the different roles of "membrane" vs "metabolic" EFAs in ethanol-stimulated prostanoid formation, and (2) may have implications for the design of therapeutic trials with EFA precursors.

Materials and Methods

Materials. [^{14}C]AA (5,8,11,14-eicosatetraenoic acid) and [^{14}C]DGLA (8,11,14-eicosatrienoic acid) (both 58 mCi/mmole) and [^{3}H]PGE_1 and [^{3}H]PGE_2 (both 28 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA. TLC plates (LK5D silica gel plates, 20 cm ht.) were obtained from the Whatman Co., Clifton, NJ. Prostaglandin standards [PGE_1, PGE_2, thromboxane (TXB)] and all other reagents were from the Sigma Chemical Co., St. Louis, MO.

Methods. Platelet-rich plasma was prepared from citrated

venous blood (30–60 cc) as previously described [21]. For pulse-labeled platelets, 5 ml of platelet-rich plasma was incubated (60 min at 37°) with 2.5 μCi [^{14}C]AA or [^{14}C]-DGLA (labeled EFAs were briefly sonicated in Krebs–Henseleit buffer prior to incubation). Following pulse labeling, platelets were pelleted, washed with Hambergs buffer, and resuspended in Krebs–Henseleit buffer. All additions to the incubation were made in this buffer. Washed platelets (non-pulse labeled) were prepared identically except for the pulse-labeling incubation.

Prostaglandin synthesis in both platelet preparations was determined during a 15-min incubation as previously described [1]. Washed resuspended (non-pulse-labeled) platelets were incubated for 15 min with test substances; [^{14}C]AA or [^{14}C]DGLA was added for the last 5 min as indicated. Pulse-labeled platelets were also incubated with test substances for 15 min; thrombin or buffer vehicle was added for the last 5 min. After the 15-min incubation, [^{3}H]-PGE_1 or [^{3}H]PGE_2 was added to monitor recovery, and the reaction was terminated by boiling for 2.5 min. Platelets were then pelleted by centrifugation, and PGE_1, PGE_2 and TXB in the supernatant fraction were separated by thin-layer chromatography developed in chloroform–methanol–acetic acid–water (90:6:1:0.75).

Labeled fatty acids, prostanoids and phospholipids from incubations with pulse-labeled platelets were extracted and separated according to Bills *et al.* [22]. Further product identification was accomplished by high performance liquid chromatography (HPLC) according to Terragno *et al.* [23], and by argentation TLC, both of which separate PGE_1 and PGE_2.

Protein was determined by the method of Lowry *et al.* [24], using bovine serum albumin as the standard.

Results

In pulse-labeled platelets (Tables 1 and 2), the following effects were observed: (1) after washing, nearly all remaining labeled [^{14}C]AA or [^{14}C]DGLA was esterified to phospholipids (Table 1), (2) minimal or no detectable prostanoids were formed under basal conditions or in incubations with aspirin or indomethacin (Tables 1 and 2), (3) addition of thrombin (3 units/tube) stimulated the release of [^{14}C]AA from membrane phospholipid (Table 1) as evidenced by an increase in free fatty acids and formation of prostanoids, (4) a concomitant decrease in [^{14}C]phospholipids was observed, (5) thrombin only slightly enhanced release of [^{14}C]DGLA and failed to stimulate formation of oxygenated products, and (6) ethanol (100 or 500 mg/100 ml) affected neither basal nor stimulated product formation in pulse-labeled platelets incubated with [^{14}C]AA or [^{14}C]DGLA (Table 2).

In parallel experiments using non-pulse-labeled platelets

Table 1. Effects of thrombin on [^{14}C]AA and [^{14}C]DGLA metabolism in pulse-labeled platelets*

	Counts per minute					
	[^{14}C]AA			[^{14}C]DGLA		
	Neutral lipids	Prostanoids	Phospholipids	Neutral lipids	Prostanoids	Phospholipids
Control	194 ± 2	23 ± 2	6861 ± 124	493 ± 10	19 ± 1	5473 ± 113
Thrombin	688 ± 40†	110 ± 6†	6482 ± 305	552 ± 14‡	25 ± 4	5592 ± 500

* Platelets from a single donor were pulse labeled with [^{14}C]AA or [^{14}C]DGLA, washed, and then briefly incubated in triplicate with or without thrombin (3 units/tube). Neutral lipids, prostanoids, and phospholipids in a total lipid extract were separated by silicic acid chromatography. Values are mean ± S.E.M. Similar data were obtained using platelets from other donors.

†, ‡ Significantly different from control (Student's *t*-test, two-tailed): † $P < 0.0005$, and ‡ $P < 0.05$.

Table 2. Effects of ethanol, thrombin, and non-steroidal anti-inflammatory agents on prostonoid synthesis in pulse-labeled platelets*

	Counts per minute			
	[^{14}C]AA pulse-labeling		[^{14}C]DGLA pulse-labeling	
	TXB	PGE_2	TXB	PGE_1
Blank conditions				
Aspirin, 250 μM	73 ± 5	65 ± 4	67 ± 1	75 ± 1
Indomethacin, 100 μM	75 ± 4	60 ± 3	80 ± 2	70 ± 3
Basal condition				
Buffer	80 ± 3	75 ± 2	65 ± 2	73 ± 2
Test conditions				
Ethanol, 100 mg/100 ml	80 ± 2	73 ± 5	83 ± 3	73 ± 2
Ethanol, 500 mg/100 ml	75 ± 5	93 ± 6	70 ± 4	67 ± 4
Thrombin, 3 units/tube	265 ± 12	87 ± 4	86 ± 2	79 ± 2
Thrombin, 3 units/tube, + ethanol, 100 mg/100 ml	260 ± 15	80 ± 3	70 ± 2	73 ± 2
Thrombin, 3 units/tube, + ethanol, 500 mg/100 ml	250 ± 8	85 ± 3	68 ± 3	76 ± 3

* Washed pulse-labeled platelets were incubated for a total of 15 min under the conditions described; thrombin (or buffer vehicle) was added for the last 5 min of the incubation. PGE_1, PGE_2, and TXB formed were separated by TLC. Values are mean ± S.E.M. from triplicate determinations. In other assays, boiled tissue or 0° blanks were identical to aspirin and indomethacin conditions.

Table 3. Effects of ethanol on prostanoid synthesis in washed resuspended platelets briefly incubated with [^{14}C]AA or [^{14}C]DGLA*

Substrate	Product	Labeled prostanoid formed (nmoles/mg protein) Ethanol concentration (mg/100 ml) 0	100	250	500
[^{14}C]AA	TXB	0.66 ± 0.02	0.69 ± 0.02	0.78 ± 0.02	0.81 ± 0.05
[^{14}C]AA	PGE_2	1.01 ± 0.14	1.02 ± 0.04	1.04 ± 0.11	0.89 ± 0.02
[^{14}C]DGLA	TXB	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.12 ± 0.02
[^{14}C]DGLA	PGE_1	1.45 ± 0.04	1.69 ± 0.05	1.65 ± 0.05	2.08 ± 0.21

* Washed resuspended platelets (not pulse-labeled) were incubated for 15 min in the presence of ethanol (0–500 mg/100 ml); [^{14}]AA (50 μM) or [^{14}C]DGLA (50 μM) was added for the last 5 min of the incubation. Prostanoids formed were separated by TLC. Data are expressed as mean ± S.E.M. of triplicate determinations. Similar results were obtained in experiments using platelets from other donors, and confirmed using HPLC or argentation TLC separations. Significant effects of ethanol were seen for TXB formation from [^{14}C]AA ($F[3, 11] = 5.22$, $P = 0.033$) and for PGE_1 formation from [^{14}C]DGLA ($F[3, 11] = 5.30$, $P = 0.026$) by one-way ANOVA.

incubated with [^{14}C]AA or [^{14}C]DGLA and test substances (Table 3), we observed the following effects: (1) there was significant basal synthesis of [^{14}C]PGE_1 in incubations with [^{14}C]DGLA and of [^{14}C]PGE_2 and [^{14}C]TXB in incubations with [^{14}C]AA, (2) ethanol significantly enhanced the formation of [^{14}C]PGE_1 from [^{14}C]DGLA, (3) in incubations with [^{14}C]AA, ethanol significantly enhanced [^{14}C]TXB synthesis, and (4) ethanol had no effect on [^{14}C]PGE_2 formation. The effect of ethanol on synthesis of [^{14}C]PGE_1 from [^{14}C]DGLA and [^{14}C]TXB from [^{14}C]AA was seen over a physiologically relevant range from 100 to 500 mg/100 ml (Table 3).

Discussion

These data are consistent with previous reports of pulse-labeled platelet release of AA and synthesis of prostanoids in the presence of thrombin [25]. It has also been shown that platelets pulse-labeled with DGLA show little capacity for either DGLA release or PGE_1 synthesis under the same conditions [25]. Ethanol affects neither basal nor thrombin-stimulated EFA metabolism under these conditions. In contrast, however, ethanol significantly increases synthesis of PGE_1 from DGLA and TXB from AA when platelets are incubated with free EFAs and ethanol. We do not know if prostanoid synthesis occurring from free EFAs is relevant to platelet function or hemostasis.

The findings reported here support the concept that free EFAs may be one of two important sources for prostanoid synthesis. Ethanol appeared to influence PG synthesis only when free precursor EFAs were present. Further, our data showing effects of ethanol on free EFA conversion to prostanoids are compatible with *in vivo* findings that small doses of EFAs markedly alter behavioral responses to ethanol. We found that ethanol had no effect on prostanoid synthesis in either [^{14}C]AA or [^{14}C]DGLA pulse-labeled platelets. Whether an analogous situation exists for different precursor pools in brain remains to be determined.

There are at least three related published studies describing effects of ethanol on prostanoid synthesis. None of these studies addresses the issue of pool-dependent synthesis; however, there are data regarding differential effects of alcohol on EFA conversion to prostanoids. Manku *et al.* [2] found that ethanol enhances formation of PGE_1 and TXB from DGLA but does not affect formation of products from AA. Pennington and Smith [3] reported that ethanol does not alter the metabolism of AA except at high ethanol concentrations. Both of these studies were carried out in non-pulse-labeled platelets. Anton *et al.* [5] reported that *in vivo* ethanol increases brain levels of PGE and TXB. Our findings with PGE_1 and PGE_2 are consistent with those of Manku *et al.*; likewise, in both our studies and those of Anton *et al.* effects of alcohol on PGE and TXB were seen. The discrepancies between our data and those of Manku *et al.* and Pennington and Smith are not readily explained except by invoking differences in species and/or what appear to be relatively minor methodological differences.

In summary, ethanol did not modify platelet prostanoid synthesis from EFAs esterified to phospholipids either under basal conditions or following phospholipase activation. However, prostanoid formation from exogenous (non-esterified) EFAs was enhanced by ethanol. Our work suggests that ethanol enhances formation of [^{14}C]PGE_1 in incubations with [^{14}C]DGLA and of [^{14}C]TXB in incubations with [^{14}C]AA; PGE_2 synthesis was not affected.

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