Thrombin-induced inositol trisphosphate production by rabbit platelets is inhibited by ethanol

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Ethanol has an inhibitory effect on some platelet functions, but the mechanisms by which it exerts this effect are not known. Using suspensions of washed platelets, we observed that ethanol (1–9 mg/ml) did not affect the aggregation of rabbit platelets stimulated with ADP (0.5–10 μ M). When platelets were prelabelled with 5-hydroxy[14 C]tryptamine, aggregation and secretion of granule contents in response to thrombin (0.01–0.10 unit/ml) were not inhibited by ethanol, but these responses to thrombin at lower concentrations (< 0.01 unit/ml) were inhibited by ethanol (2–4 mg/ml). Platelets were prelabelled with [3 H]inositol so that increases in inositol phosphates upon stimulation could be assessed by measuring the amount of label in these compounds. ADP-induced increases in IP (inositol phosphate) and IP₂ (inositol bisphosphate) were not affected by ethanol. IP₃ (inositol trisphosphate) was not changed by ADP or ethanol. Although ethanol did not affect the increases in IP, IP₂ and IP₃ caused by stimulation of platelets with thrombin at concentrations greater than 0.01 unit/ml, ethanol did inhibit the increases observed at 2 and 3 min in these inositol phosphates caused by lower concentrations of thrombin (< 0.01 unit/ml). Since ADP did not cause formation of IP₃ in rabbit platelets, and since no thromboxane B₂ was detected in platelets stimulated with the lower concentrations of thrombin, it is unlikely that the inhibitory effect of ethanol in IP₃ formation was due to effects on further stimulation of platelets by released ADP or by thromboxane A₂. Ethanol may inhibit platelet responses to thrombin by inhibiting the production of the second messenger, IP₃.

INTRODUCTION

Evidence from epidemiological studies indicates that moderate consumption of alcoholic beverages has a protective effect against coronary heart disease [1–5]. Several reasons have been proposed for this beneficial effect of alcohol, including direct effects of ethanol on platelets [6]. Ethanol has been reported to inhibit platelet responses to aggregating agents *in vitro* [7–10], but the mechanism by which ethanol exerts its inhibitory effect is not known. Fluidity of cell membranes is increased by ethanol [11], and it may well be that an alteration in platelet membrane fluidity by ethanol is involved in its inhibitory effect on platelet function. Production of the arachidonic acid metabolites malonyldialdehyde and TXB₂ by platelets stimulated with thrombin or collagen is inhibited by ethanol [12–14], and it has been suggested that ethanol inhibits Ca²⁺-activated phospholipase A₂ in platelets [15,16].

An early response of many types of cells to stimulation is the hydrolysis of membrane-bound PIP₂ by phospholipase C to IP₃ and 1,2-diacylglycerol [17–19]. IP₃ is formed by thrombin-stimulated platelets [20–23], and has been shown to release Ca²⁺ from intracellular membranes of platelets [24] and to induce secretion of granule contents from permeabilized platelets [25,26]. In contrast, stimulation of rabbit platelets with ADP, which induces very little secretion of granule contents [27], is not associated with the formation of IP₃ [23]. In the

present study, we have examined the effect of ethanol, at a concentration that is physiologically tolerable, on ADP- or thrombin-induced aggregation, secretion of granule contents, and production of inositol phosphates by rabbit platelets, to determine whether the inhibitory effect of ethanol on platelet function is associated with an effect of ethanol on the production of IP₃.

MATERIALS AND METHODS

Materials

Materials were obtained from the following suppliers: ADP from Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine thrombin (topical) from Parke-Davis, Scarborough, Ont., Canada; ethanol from Consolidated Alcohols, Toronto, Ont., Canada; imipramine from Geigy Canada, Dorval, Que., Canada; and albumin (fraction V) from Boehringer Mannheim, Dorval, Que., Canada. [3 H]Inositol (14 Ci/mmol) was purchased from American Radiolabeled Chemicals, St. Louis, MO, U.S.A., 5-hydroxy[14 C]tryptamine creatinine sulphate ('[14 C]serotonin'; 60 mCi/mmol) from Amersham Corp., Oakville, Ont., Canada, and a radioimmunoassay kit (NEK-007) for TXB₂ from NEN Canada, Lachine, Que., Canada. The sensitivity of the assay is such that an increase of 0.5 ng of TXB₂/10⁹ platelets in a stimulated sample compared with its unstimulated control can be detected reliably.

Abbreviations used: IP, IP₂, IP₃, inositol phosphate, bisphosphate and trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; TXA₂, TXB₂, thromboxane A₂ and B₂.

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Preparation of prelabelled washed platelets from rabbits

Rabbit blood was collected into acid-citrate-dextrose [28], and suspensions of washed platelets were prepared by the method of Ardlie *et al.* [29,30]. For studies on the effects of ethanol on platelet aggregation and secretion of the contents of the amine-storage granules, platelets were labelled in the first washing solution with 1 μ Ci of [14 C]serotonin for the platelets from each rabbit (10 min at room temperature), and, after a washing step, were resuspended at a count of 0.5×10^9 platelets/ml in Tyrode solution containing 0.35% albumin, 5 μ M-imipramine and apyrase (the concentration of apyrase, prepared by the method of Molnar & Lorand [31], to be used was determined as described elsewhere [32]). Platelet suspensions were incubated at 37 °C for at least 20 min before testing.

Alternatively, for studies of the accumulation of labelled inositol phosphates, platelets (2×10^9 /ml) were labelled in the first washing solution with 20 μ Ci of [3 H]inositol/ml of platelet suspension for 1 h at 37 °C. After two washing steps to remove unincorporated label, the platelets were resuspended at a count of 0.5×10^9 /ml in Tyrode solution containing 0.35% albumin, apyrase and 20 mM-LiCl (the amount of NaCl added to Tyrode solution was decreased to maintain an osmolarity of 290 mosM). The platelet suspensions were incubated at 37 °C for 1 h; in this period of time, the Li⁺ concentration in the platelets approaches 80% of the concentration in the medium [33].

Stimulation of prelabelled platelets and measurement of accumulation of inositol phosphates

Platelets in an aggregometer cuvette were stirred in a Payton Aggregation Module (Payton Associates, Scarborough, Ont., Canada) for 30 s. Modified Tyrode solution (lacking glucose), or ethanol diluted with modified Tyrode solution, was added, stirring was continued for 2 min, and platelets were stimulated with ADP or thrombin. The changes in light transmission through the stirred suspensions of platelets were recorded. At 3 min after addition of agonist to platelets prelabelled with [14 C]serotonin, the platelet suspensions were rapidly transferred to Eppendorf centrifuge tubes, and centrifuged at 12000 *g* for 1 min. Radioactivity in samples of the supernatant fluid was measured to determine secretion of 14 C-labelled material [34], and the supernatant samples were also used for radioimmunoassays of TXB₂. The amounts of [14 C]serotonin and TXB₂ in the supernatants from unstimulated samples were subtracted in calculating the percentages of [14 C]serotonin secreted and the amounts of TXB₂ formed by the platelets upon stimulation. At 2 or 3 min after addition of agonist to platelets prelabelled with [3 H]inositol, the reaction was terminated by addition of ice-cold trichloroacetic acid (final concn. 10%, w/v), and, after mixing, the inositol phosphates were recovered and fractionated by anion-exchange chromatography as previously described [22,23].

Analysis of data

For determination of the amount of label in the inositol phosphates, values in individual experiments were standardized to a total incorporation of [3 H]inositol of 2×10^5 d.p.m./ 10^9 platelets [23]. Data were analysed as described in the legend of Table 1, by paired *t* tests.

RESULTS

Effects of ethanol on platelet aggregation and secretion of granule contents induced by ADP or thrombin

Stimulation of rabbit platelets with ADP, which caused shape change and reversible aggregation, as determined by changes in light transmission through stirred suspensions of washed platelets, did not induce measurable secretion of 14 C-labelled material from platelets prelabelled in their amine-storage granules with [14 C]serotonin. Ethanol (1–9 mg/ml) had no effect on platelet aggregation induced by ADP (0.5–10 μ M); Fig. 1 shows the lack of effect of ethanol at 4 mg/ml on aggregation induced by 5 μ M-ADP.

Stimulation of platelets with a range of thrombin concentrations (0.005–0.1 unit/ml) caused shape change, aggregation, and secretion of the contents of amine-storage granules. Ethanol (2–4 mg/ml) inhibited aggregation of and secretion by platelets in response to low concentrations of thrombin (< 0.01 unit/ml). Detectable amounts of TXB₂ were not produced by either control platelets (0.0 ± 0.2 ng of TXB₂/ 10^9 platelets) or platelets treated with ethanol (0.0 ± 0.2 ng/ 10^9 platelets; means $\pm$ S.E.M., *n* = 12) 3 min after addition of the low concentrations of thrombin. The inhibitory effects of ethanol on aggregation and secretion were not observed when higher concentrations of thrombin were used; Fig. 2 shows the inhibitory effect of ethanol (2 and 4 mg/ml) on aggregation and secretion in response to 0.008 unit of

Fig. 1. Recordings of light transmission through stirred platelet suspensions stimulated with 5 μ M-ADP in the absence and presence of ethanol (4 mg/ml)

Platelets were stirred with or without ethanol for 2 min before addition of ADP, indicated by the arrows. No significant amounts of secretion of 14 C-labelled material from the prelabelled platelets were detected 3 min after addition of ADP. The aggregation tracings are representative of the results of three experiments. The lack of effect of ethanol on ADP-induced aggregation of platelets was observed over a wide range of ADP and ethanol concentrations (0.5–10 μ M and 1–9 mg/ml respectively).

Fig. 2. Recordings of light transmission through stirred platelet suspensions stimulated with (a) low (0.008 unit/ml) or (b) higher (0.050 unit/ml) concentrations of thrombin, in the absence or presence of ethanol

Platelets were stirred with or without ethanol for 2 min before addition of thrombin (THR), indicated by the arrows. The extent (% of total radioactivity in the platelets) of secretion of ^{14}C -labelled material from the prelabelled platelets was determined 3 min after addition of thrombin and is given in (a) under the heading and in (b) in the boxes. The results are representative of three experiments, in which ethanol (2–4 mg/ml) inhibited aggregation and secretion by platelets in response to low concentrations of thrombin (0.005–0.01 unit/ml), but not higher concentrations of thrombin (> 0.01 unit/ml).

Table 1. Effect of ethanol (4 mg/ml) on the labelling of IP_1 , IP_2 and IP_3 produced by platelets prelabelled with $[^3\text{H}]$ -inositol and stimulated with $5 \mu\text{M}$ -ADP for 2 min

'Increase due to stimulation' is the difference between the ADP-treated sample or the ethanol-and-ADP-treated sample and the paired unstimulated sample to which only Tyrode solution or ethanol and Tyrode solution was added. 'Change due to ethanol' is the absolute value of the difference between the ADP-stimulated sample (with unstimulated sample subtracted) and the ADP-stimulated sample to which ethanol was added (with unstimulated sample with ethanol subtracted). Data are expressed as the mean differences $\pm$ S.E.M. for seven experiments. Significances were tested with paired t tests: * $P < 0.01$; † $P < 0.001$.

Inositol phosphate	Increase due to stimulation (d.p.m./ 10^9 platelets)	Increase due to stimulation in the presence of ethanol (d.p.m./ 10^9 platelets)	Change due to ethanol (d.p.m./ 10^9 platelets)
IP_1	$1995 \pm 504^*$	$2245 \pm 529^*$	250 ± 180
IP_2	$1353 \pm 178^\dagger$	$1488 \pm 198^\dagger$	135 ± 60
IP_3	62 ± 47	68 ± 43	6 ± 6

Effects of ethanol on production of inositol phosphates by platelets stimulated with ADP or thrombin

Platelet phosphoinositides were prelabelled with $[^3\text{H}]$ -inositol, and platelets were studied in the presence of 20 mM- Li^+ , which inhibits the degradation of inositol phosphate to inositol and phosphate [35]. Control experiments showed that the presence of Li^+ at this concentration had no detectable effect on platelet aggregation and secretion. When platelets were stimulated with $5 \mu\text{M}$ -ADP and the reaction was terminated after 2 min, no significant amount of label was detected in IP_3 , although there were substantial amounts of label in IP_1 and IP_2 (Table 1). Ethanol (4 mg/ml), which did not affect platelet aggregation in response to stimulation by ADP, did not significantly change the amounts of label in IP_1 , IP_2 or IP_3 (Table 1).

At 2 or 3 min after stimulation of platelets with low or high concentrations of thrombin, there were significant increases in the amounts of label in all of the inositol phosphates, IP_1 , IP_2 and IP_3 (Tables 2 and 3). This was also observed in the presence of ethanol (4 mg/ml), with one exception: with the lower concentrations of thrombin, the amount of label in IP_3 was not significantly increased in the presence of ethanol. Ethanol inhibited platelet aggregation and the increase in all the inositol phosphates caused by the lower concentrations of thrombin (Tables 2a and 3a). However, at higher concentrations of thrombin, platelet aggregation was not affected by ethanol, and ethanol did not cause significant changes in the amounts of label in the inositol phosphates, except IP_2 at 3 min (Tables 2b and 3b).

DISCUSSION

These experiments show that the inhibitory effect of ethanol on platelet aggregation and secretion of granule contents in response to low concentrations of thrombin

thrombin/ml (Fig. 2a) and the lack of effect of ethanol at 4 mg/ml on aggregation and release induced by 0.05 unit of thrombin/ml (Fig. 2b).

Table 2. Effect of ethanol (4 mg/ml) on the labelling of IP, IP₂ and IP₃ produced by platelets prelabelled with [³H]inositol and stimulated with thrombin for 2 min

'Increase due to stimulation' and 'Change due to ethanol' were calculated as described in the legend of Table 1. Data are expressed as the mean differences $\pm$ S.E.M. for five experiments (lower concentrations of thrombin, *a*) and six experiments (higher concentrations of thrombin, *b*). Inhibition of platelet aggregation by ethanol was observed at the lower range of concentrations of thrombin (0.0065–0.009 unit/ml), but was not observed at the higher range of concentrations of thrombin (0.0125–0.0175 unit/ml). Significances were tested with paired *t* tests. All increases due to stimulation with thrombin in the absence or presence of ethanol were significantly different from zero, except the increase in IP₃ in the presence of ethanol, at lower concentrations of thrombin: **P* < 0.05; †*P* < 0.025; ‡*P* < 0.005; §*P* < 0.001.

Thrombin concns.	Inositol phosphate	Increase due to stimulation (d.p.m./10 ⁹ platelets)	Increase due to stimulation in the presence of ethanol (d.p.m./10 ⁹ platelets)	Change due to ethanol (d.p.m./10 ⁹ platelets)
(a) Lower (0.0065–0.009 unit/ml)	IP	1710 $\pm$ 269‡	1260 $\pm$ 163‡	450 $\pm$ 136*
	IP ₂	3413 $\pm$ 376§	1876 $\pm$ 226‡	1536 $\pm$ 463*
	IP ₃	338 $\pm$ 76†	97 $\pm$ 44	241 $\pm$ 73*
(b) Higher (0.0125–0.0175 unit/ml)	IP	2421 $\pm$ 462‡	2250 $\pm$ 290§	171 $\pm$ 206
	IP ₂	4171 $\pm$ 774‡	4148 $\pm$ 740‡	24 $\pm$ 161
	IP ₃	213 $\pm$ 59†	126 $\pm$ 37†	87 $\pm$ 52

Table 3. Effect of ethanol (4 mg/ml) on the labelling of IP, IP₂ and IP₃ produced by platelets prelabelled with [³H]inositol and stimulated with thrombin for 3 min

'Increase due to stimulation' and 'Change due to ethanol' were calculated as described in the legend of Table 1. Data are expressed as the mean differences $\pm$ S.E.M. for nine experiments (lower concentrations of thrombin, *a*) and four experiments (higher concentrations of thrombin, *b*). Inhibition of platelet aggregation by ethanol was observed at the lower range of concentrations of thrombin (0.0055–0.010 unit/ml), but was not observed at the higher range of concentrations of thrombin (0.012–0.015 unit/ml). Significances were tested with paired *t* tests. All increases due to stimulation with thrombin in the absence or presence of ethanol were significantly different from zero, except the increase in IP₃ in the presence of ethanol, at lower concentrations of thrombin: **P* < 0.05; †*P* < 0.01; ‡*P* < 0.005; §*P* < 0.001.

Thrombin concns.	Inositol phosphate	Increase due to stimulation (d.p.m./10 ⁹ platelets)	Increase due to stimulation in the presence of ethanol (d.p.m./10 ⁹ platelets)	Change due to ethanol (d.p.m./10 ⁹ platelets)
(a) Lower (0.0055–0.010 unit/ml)	IP	2792 $\pm$ 437§	1764 $\pm$ 196§	1028 $\pm$ 273†
	IP ₂	3171 $\pm$ 414§	1180 $\pm$ 243‡	1992 $\pm$ 260§
	IP ₃	281 $\pm$ 65‡	117 $\pm$ 56	165 $\pm$ 46†
(b) Higher (0.012–0.015 unit/ml)	IP	3009 $\pm$ 139§	3107 $\pm$ 126§	99 $\pm$ 211
	IP ₂	4289 $\pm$ 452§	4912 $\pm$ 371§	623 $\pm$ 161*
	IP ₃	520 $\pm$ 149*	516 $\pm$ 148*	3 $\pm$ 44

is associated with a decreased labelling of IP₃ produced by platelets prelabelled with [³H]inositol. IP₃ is involved in the mobilization of Ca²⁺ from non-mitochondrial stores in cells [19,36] and, in permeabilized platelets, has been shown to trigger the rapid release of Ca²⁺ from intracellular stores, to stimulate platelet aggregation, secretion of amine-storage-granule contents, and formation of thromboxane from arachidonic acid [25,26,37]. However, in the present study, the concentrations of thrombin used were too low to cause thromboxane formation, although they caused aggregation, secretion of amine-storage-granule contents and an increase in labelled IP₃.

Others have shown that ethanol inhibits thromboxane production by stimulated platelets [12–14], and Fenn and associates [15] have previously suggested that ethanol inhibits platelet function by inhibiting Ca²⁺-activated phospholipase A₂, the enzyme that is responsible for mobilization of arachidonic acid from platelet membrane phospholipids. However, the results of our other studies using aspirin-treated platelets [38] and the present study indicate that ethanol has an additional inhibitory effect on platelets, in that it can inhibit thrombin-induced platelet responses (aggregation, secretion and the production of IP₃) that can occur without thromboxane production. Thrombin induces platelet aggregation by a

pathway independent of secretion of ADP and the formation of TXA₂, although these agonists do potentiate the effects of lower concentrations of thrombin to a very minor extent [39]. An inhibitory effect of ethanol on thromboxane production by the platelets stimulated with low concentrations of thrombin can be ruled out as a reason for the observed decreased labelling of IP₃, for two reasons. Firstly, platelets stimulated with low concentrations of thrombin did not form detectable amounts of thromboxane in either the absence or the presence of ethanol. Secondly, ethanol also inhibits aggregation and secretion by rabbit platelets pretreated with aspirin (so they cannot form thromboxane) and stimulated with low concentrations of thrombin [38]. Although ethanol inhibited secretion of the contents (which include ADP) of storage granules of platelets stimulated with low concentrations of thrombin, stimulation of rabbit platelets with ADP is not associated with the formation of IP₃ ([23]; the present study). It therefore appears that the decreased labelling of IP₃ produced by platelets prelabelled with [³H]inositol that is associated with an inhibitory effect of ethanol on platelet aggregation and secretion of granule contents in response to low concentrations of thrombin is due to an inhibition by ethanol of the thrombin-induced pathway of platelet aggregation that is independent of released ADP or thromboxane formation.

Whether ethanol exerts its effect on IP₃ production by a direct inhibition of the activity of phospholipase C, the enzyme that catalyses hydrolysis of PIP₂ to IP₃ and 1,2-diacylglycerol, or by inhibition of the activation of phospholipase C is not known. It is possible, for example, that ethanol, by virtue of its membrane-perturbing, or fluidizing, property [11], affects the number or affinity of thrombin receptors on the platelet surface. Tandon and associates [40] have shown that platelets with increased membrane fluidity, brought about by depletion of cholesterol from the membranes, have a decreased number of thrombin receptors and a decreased aggregation response to thrombin.

The decreased labelling of IP₃ that is associated with the inhibitory effect of ethanol on platelet aggregation and secretion in response to low concentrations of thrombin is accompanied by significantly less increase in the labelling of IP₂ and IP. These two compounds have no known second-messenger activity, and may be formed by hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol, respectively, by phospholipase C and/or by dephosphorylation of IP₃. In considering these possibilities, the effects of ethanol on rabbit platelets stimulated with ADP are relevant. ADP causes increased labelling of IP and IP₂, but not IP₃, at times ranging from 10 to 120 s, as shown by the observations reported in the present paper and previously [23]. Thus the way in which ADP affects the production of inositol phosphates appears to differ from the way in which thrombin acts, because thrombin causes increased labelling of IP, IP₂ and IP₃. Since ethanol did not affect the increased labelling of IP and IP₂ in response to ADP, but did inhibit the increase in the labelling of IP and IP₂ in response to low concentrations of thrombin, it seems likely that ethanol inhibits the formation of their common precursor, IP₃.

Ethanol did not affect platelet aggregation in response to ADP, nor did it alter the ADP-induced increased labelling of the inositol phosphates. In agreement with

our earlier results [23], there was no significant increase in labelling of IP₃ in rabbit platelets stimulated to aggregate in response to ADP. There have been contradictory reports concerning whether human platelets stimulated with ADP form significant amounts of IP₃ [41–43]. There are a number of circumstances in which ADP stimulation of human platelets causes thromboxane formation and secretion of granule contents, confounding the interpretation of studies with human platelets. Since rabbit platelets do not form thromboxanes and undergo very little secretion of granule contents when stimulated with ADP [27], our results are only comparable with studies with human platelets aggregated by ADP under conditions in which thromboxane formation and secretion of granule contents do not occur to an appreciable extent.

In accordance with the observation in our experiments that ethanol can inhibit thrombin-induced production of inositol phosphates, Gonzales and associates [44] have reported that ethanol inhibits inositol phosphate production by slices taken from various regions of rat brains. Thus the effect of ethanol on inositol phospholipid metabolism may be a generalized one in various cells and tissues.

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