Biochem. J. (1990) 269, 489–497 (Printed in Great Britain)

Ethanol inhibits thrombin-induced secretion by human platelets at a site distinct from phospholipase C or protein kinase C

Christine BENISTANT and Raphael RUBIN*
Department of Pathology and Cell Biology, Jefferson Medical College, Philadelphia, PA 19107, U.S.A.

Ethanol is known to inhibit the activation of platelets in response to several physiological agonists, but the mechanism of this action is unclear. The addition of physiologically relevant concentrations of ethanol (25–150 mM) to suspensions of washed human platelets resulted in the inhibition of thrombin-induced secretion of 5-hydroxy[^{14}C]tryptamine. Indomethacin was included in the incubation buffer to prevent feedback amplification by arachidonic acid metabolites. Ethanol had no effect on the activation of phospholipase C by thrombin, as determined by the formation of inositol phosphates and the mobilization of intracellular Ca^{2+}. Moreover, ethanol did not interfere with the thrombin-induced formation of diacylglycerol or phosphatidic acid. Stimulation of platelets with phorbol ester (5–50 nM) resulted in 5-hydroxy[^{14}C]tryptamine release comparable with those with threshold doses of thrombin. However, ethanol did not inhibit phorbol-ester-induced secretion. Ethanol also did not interfere with thrombin- or phorbol-ester-induced phosphorylation of myosin light chain (20 kDa) or a 47 kDa protein, a known substrate for protein kinase C. By electron microscopy, ethanol had no effect on thrombin-induced shape change and pseudopod formation, but prevented granule centralization and fusion. The results indicate that ethanol does not inhibit platelet secretion by interfering with the activation of phosphoinositide-specific phospholipase C or protein kinase C by thrombin. Rather, the data demonstrate an inhibition of a Ca^{2+}-mediated event such as granule centralization.

INTRODUCTION

Ethanol interferes with platelet activation in response to several physiological agonists [1–7]. Platelets isolated from chronic alcoholics are generally hypoaggregable, and they regain normal function only over a period of several days of abstinence [6,7]. Similar findings have been described in non-alcoholics who have ingested ethanol [1,8,9]. Ethanol also inhibits platelet aggregation and secretion *in vitro* [2–5]. The inhibitory effect of ethanol is thought to contribute to the bleeding tendencies in chronic alcoholics [1]. Moreover, ethanol-induced platelet defects have been invoked as an explanation for its apparent protective effect on coronary heart disease [10].

The mechanism by which ethanol interferes with platelet function is unclear. Ethanol alters membrane fluidity, and can affect several membrane-associated enzymic and transport processes, presumably by affecting membrane–protein interactions [11–14]. In view of these physical effects of ethanol, recent studies have focused on the effect of ethanol on membrane-associated signal-transduction pathways in platelets and other cells [15–19]. Ethanol could affect one or more of several interrelated signal-generating processes in platelets. The activation of phospholipase A_2 leads to the production of arachidonic acid (AA), which can be metabolized by cyclo-oxygenase to stimulatory endoperoxides and thromboxane A_2 (TXA_2) [20]. Studies from our laboratory [2] and others [4,21] have presented evidence that ethanol inhibits phospholipase A_2. For example, we have recently shown in studies of [^{3}H]AA-labelled platelets that the inhibition of collagen- and Ca^{2+}-ionophore-induced platelet activation by ethanol is associated with a decrease in AA formation [2]. Moreover, the re-addition of exogenous AA restores platelet aggregation in the presence of ethanol.

A primary stimulatory event after the binding of agonists to human platelets is the activation of phosphoinositide-specific phospholipase C, a process which leads to the production of $InsP_3$ and diacylglycerol (DG) (see ref. [22] for review). $InsP_3$ mobilizes intracellular Ca^{2+}, which in turn stimulates the phosphorylation of myosin light chain (20 kDa) and promotes cytoskeleton reorganization. DG production leads to the activation of protein kinase C and the subsequent phosphorylation of a 40–47 kDa protein. The concerted action of these diverse signals leads to a synergistic functional response.

The effect of ethanol on phospholipase C is unclear. It has been reported that ethanol can inhibit thrombin-induced $InsP_3$ production in rabbit platelets [23]. In studies of human platelets [2], ethanol did not inhibit total inositol phosphate formation in response to collagen under conditions where TXA_2 formation was inhibited by indomethacin. Moreover, acute exposure of human platelets [16] and isolated rat hepatocytes [15] to high concentrations of ethanol actually stimulates phosphoinositide-specific phospholipase C.

In the present study, we investigate the effect of ethanol on thrombin- and phorbol-ester-induced platelet secretion in the overall context of signal generation. Studies were performed in the presence of indomethacin, an agent which minimizes the formation of stimulatory AA metabolites. The results indicate that ethanol inhibits thrombin-induced secretion, but has no effect on the activation of phospholipase C and protein kinase C. The data support a role for the direct interference by ethanol with a Ca^{2+}-mediated event such as granule movement that occurs after the activation of phospholipase C and protein kinase C.

Abbreviations used: AA, arachidonic acid; TX, thromboxane; DG, diacylglycerol; 5-HT, 5-hydroxytryptamine; PA, phosphatidic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concn.; PMA, phorbol myristate acetate.

* To whom reprint requests should be addressed.

EXPERIMENTAL

Materials

Thrombin and ionomycin (free acid) were obtained from Sigma. AA was purchased from Chronolog Co. (Havertown, PA, U.S.A.). [5,6,8,9,11,12,14,15-^{3}H]AA (135 Ci/mmol), carrier-free $H_3{}^{32}PO_4$ and 5-hydroxy[2-^{14}C]tryptamine (5-[^{14}C]HT) were purchased from Amersham Corp. *myo*-[2-^{3}H]Inositol (15 Ci/mmol in 95% ethanol) was obtained from American Radiolabeled Chemicals. Before use of the [^{3}H]inositol, the ethanol was removed by evaporation and the radiochemical was redissolved in water and treated with a small quantity of Dowex 1-X8 anion-exchange resin (formate form, 200–400 mesh; Bio-Rad) to remove any negatively charged impurities. A water-soluble preparation of indomethacin was obtained from Chiesi Pharmaceuticals, Parma, Italy. Fura2-AM was purchased from Calbiochem. Other chemicals and biochemicals of the highest purity commercially available were purchased from Sigma or from Fisher Scientific.

Preparation of platelets, isotopic labelling and incubation conditions

Blood was obtained from normal human volunteers who had been free from medication for at least 10 days. The sample was anti-coagulated with $\frac{1}{6}$ vol. of acid/citrate/dextrose (ACD; 1.5 g of citric acid, 2.5 g of sodium citrate and 2 g of glucose per 100 ml of water). Platelet-rich plasma was obtained by centrifugation of whole blood at 200 *g* for 15 min at room temperature, and was then centrifuged at 800 *g* for 20 min. The platelet pellet was resuspended in 2 ml of Hepes buffer, containing 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes (pH 7.4 at 37 °C), 10 mM-glucose and 0.2% BSA (fatty-acid-free). For isotopic labelling, the platelet suspension was incubated at 37 °C in a shaking water bath in the presence of 0.5 mM-EGTA and 10 μM-indomethacin with 10 μCi of [^{3}H]AA (1 h), 0.3 mCi of [^{32}P]P_i (90 min), 0.4 mCi of [^{3}H]inositol (3 h), or 2 μCi of 5-[^{14}C]HT (15 min). Indomethacin (10 μM) was included in the incubation medium unless it was to be excluded in subsequent incubations. For fura2 loading, platelets were incubated with 4 μM-fura2-AM for 30 min. After labelling, the platelets were washed in 35 ml of ACD-treated Hepes buffer (pH 6.4). The platelet pellet was resuspended in Hepes buffer at a final platelet concentration of $(5–8) \times 10^8$/ml.

Unless otherwise specified, incubations were carried out in a water bath at 37 °C. Platelets were preincubated for 5 min before addition of agonists. For analysis of 5-[^{14}C]HT release, 5 mM-EDTA was added to the platelet suspension and the samples were stored on ice. The platelets were then centrifuged at 8000 *g* for 1 min, and samples of the supernatants were taken for determination of released 5-[^{14}C]HT by liquid-scintillation counting. Total 5-[^{14}C]HT content was determined in samples before centrifugation, and was 3125 ± 318 c.p.m. The amount of radioactivity released by centrifugation amounted to less than 5% of the total, and values were corrected for this amount. For analysis of inositol phosphates and phosphatidic acid (PA), samples were quenched in 3.75 vol of chloroform/methanol/HCl (40:20:1, by vol.). For measurement of [^{3}H]DG and [^{3}H]AA and metabolites, samples were quenched in 3.75 vol. of ice-cold chloroform/methanol (1:2, v/v). For measurement of [^{32}P]phosphoproteins, samples were added to an equal volume of 2 × Laemmli sample buffer [24].

Intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was determined in fura2-loaded platelets as described previously [16]. Samples (2 ml) of fura2-loaded platelets were incubated at 37 °C in the cuvette of a PTI Deltascan dual-excitation-wavelength spectrofluorimeter at dual excitation wavelengths of 340 nm and 380 nm, with an emission wavelength of 510 nm. Other details were as described by Grynkiewicz *et al.* [25].

Assays

[^{3}H]Inositol phosphates. For this, 1.25 vol. each of chloroform and water were added to the chloroform/methanol extract, and the sample was centrifuged. The upper methanol phase was neutralized with 2 M-KOH/10 mM-Mops. The radioactivities of the individual inositol phosphates were determined after separation by anion-exchange chromatography and liquid-scintillation counting as described elsewhere [17].

[^{3}H]DG. For this, 1.25 vol. each of chloroform and water were added to the chloroform/methanol extract. After centrifugation, the chloroform layer was taken, and the lipids were separated on silica-gel-60 plastic-backed t.l.c. plates (20 cm × 20 cm; EM reagents) with the solvent system benzene/diethyl ether/conc. NH_3 (500:400:1, by vol.). The identity of 1,2-DG was determined by co-migration with authentic 1,2-DG standard (Sigma). [^{3}H]DG was cut out and the radioactivity determined by liquid-scintillation counting in Budget-Solve scintillation fluid (Research Products International). Total [^{3}H]AA release and [^{32}P]PA formation were analysed by t.l.c. and liquid-scintillation counting as described previously [2].

[^{32}P]Phosphoproteins. Samples were boiled for 3 min and the proteins separated on SDS/11%-polyacrylamide gels by the method of Laemmli [24]. The gels were stained with Coomassie Brilliant Blue, dried, autoradiographed, and the bands corresponding to the 20 kDa and 47 kDa proteins were cut out and their radioactivities determined by liquid-scintillation counting.

Electron microscopy

Platelets were fixed in phosphate-buffered 2% glutaraldehyde. The samples were centrifuged and the pellets washed three times in phosphate buffer. The platelets were post-fixed in 1% OsO_4, washed, and dehydrated in graded concentrations of ethanol. Samples were then imbedded in Spurr's medium and processed for examination with a JEOL 100cx electron microscope.

Statistics

All experiments were performed at least three times with different donors. Results are expressed as the means ± S.E.M. for n determinations. Statistical significance was determined by Student's t test.

RESULTS

The stimulatory effects of several physiological agonists on human platelets can be amplified by the metabolites of AA, which, in turn, is generated by the action of phospholipase A_2 [20]. In a previous study [2], we have demonstrated that ethanol interferes with the mobilization of AA in response to collagen, and this effect may be related to the inhibition of collagen-induced platelet activation. Ethanol (150 mM) also inhibited AA formation by about 50% in response to thrombin (0.05 unit/ml) [2], but the significance of this inhibition as it relates to platelet function has not been defined. The experiment of Table 1 further examines these relationships in platelets stimulated by thrombin and AA. Addition of a high concentration of AA (50 μM) to a stirred suspension of platelets resulted in a 41% release of 5-[^{14}C]HT. Ethanol (150 mM) inhibited this response by about 45%. Preincubation of platelets with the cyclo-oxygenase inhibitor indomethacin completely prevented AA-induced secretion. Moreover, in [^{3}H]AA-labelled platelets, indomethacin

Table 1. Effect of ethanol and indomethacin on 5-[^{14}C]HT secretion after stimulation with AA or thrombin

Samples (0.5 ml) of 5-[^{14}C]HT-labelled platelets were stirred in aggregometer tubes at 37 °C for 3 min with the indicated addition in the absence or presence of ethanol (150 mM). Secretion of 5-[^{14}C]HT was measured as described in the Experimental section, and is expressed as the percentage of total 5-[^{14}C]HT. Data are means ± S.E.M. of a representative experiment performed in triplicate.

	5-[^{14}C]HT secretion (% of total)	
Addition	(−) ethanol	(+) ethanol
AA (50 μM)	41±5	24±8
AA + indomethacin (10 μM)	1.6±0.8	–
Thrombin (0.025 unit/ml) + indomethacin	22±2.5	2.4±0.4
Thrombin + AA + indomethacin	24.3±4	4.9±0.8

completely prevented the formation of [^{3}H]TXB_2 (results not shown). Thus these data demonstrate that the stimulatory effect of AA is completely dependent on its subsequent metabolism.

In the presence of indomethacin, ethanol almost completely prevented secretion in response to thrombin (0.025 unit/ml). Addition of AA did not enhance the response to thrombin, and ethanol still inhibited secretion by over 80%. Thus these data indicate that ethanol inhibits thrombin-induced secretion by a mechanism that is not related to its inhibitory effect on AA formation. The inhibitory action of ethanol was also independent of autocrine stimulation by ADP. In the presence of both indomethacin and the ADP-receptor antagonist FSBA (100 μM; Sigma) [26] or the ADP-scavenger system phosphocreatine (5 mM)/creatine kinase (10 units/ml), ethanol inhibited secretion induced by 0.05 units of thrombin/ml by 75% and 56% respectively (means of two experiments). In the presence of 1 μM-imipramine, ethanol still inhibited secretion induced by thrombin (0.025 unit/ml) by 69±9% ($n = 3$). Therefore the effect of ethanol was also not due to increased re-uptake of 5-HT.

In the following experiments, we investigated this AA-independent mechanism of inhibition by ethanol; the studies were all performed in the presence of indomethacin. The effect of ethanol on the concentration-dependence of thrombin for platelet secretion is shown in Fig. 1. Ethanol had no effect on the basal release of 5-[^{14}C]HT. Secretion in response to a threshold concentration of thrombin (0.025 units/ml) was completely prevented by ethanol, and the half-maximal response was inhibited by about 50%. Higher concentrations of thrombin overcame the inhibitory effect of ethanol, and secretion induced by 0.2 unit of thrombin/ml was unaffected by ethanol. In a stirred suspension of platelets, ethanol also inhibited thrombin-induced ATP secretion in a manner comparable with 5-[^{14}C]HT secretion, as measured in the luminescence channel of a Chronolog lumi-aggregometer (results not shown).

The concentration-dependence of ethanol for the inhibition of thrombin-induced secretion is shown in Fig. 2. Significant ($P < 0.001$) differences from control incubations were observed with ethanol concentrations of 25–50 mM. These concentrations are tolerated physiologically, and are commonly found in human alcoholics [27]. The inhibitory effect of ethanol increased linearly up to 150 mM-ethanol. There was no lysis of platelets over this concentration range, as measured by the release of lactate dehydrogenase.

It has been suggested elsewhere [23] that ethanol may inhibit thrombin-induced secretion by interference with the activation of phospholipase C. The effect of ethanol on the concentration-dependence of thrombin for inositol phosphate formation in [^{3}H]inositol-labelled platelets is illustrated in Fig. 3. LiCl (10 mM) was included in the incubation medium to prevent the breakdown of Ins$P$ to *myo*-inositol. Inositol phosphate production was demonstrable at concentrations of thrombin of 0.01–0.1 unit/ml. Ethanol had no effect on basal or thrombin-stimulated levels of InsP_3, InsP_2 and Ins$P$.

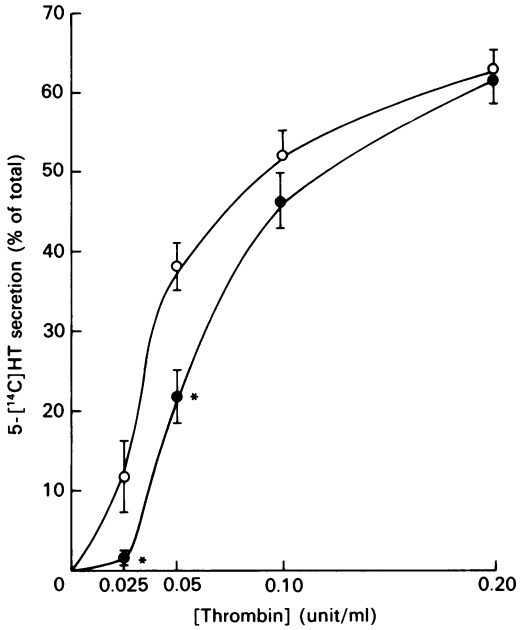


Fig. 1. Effect of ethanol on thrombin-induced secretion of 5-[^{14}C]HT

5-[^{14}C]HT-labelled platelets were incubated at 37 °C for 3 min with the indicated concentrations of thrombin in the absence (○) or presence (●) of ethanol (150 mM). Ethanol was added 2 min before the thrombin. Each point is the mean ± S.E.M. of 5–8 separate experiments, with each measurement expressed as the percentage of total 5-[^{14}C]HT, as described in the Experimental section: $*P < 0.05$ compared with thrombin alone.

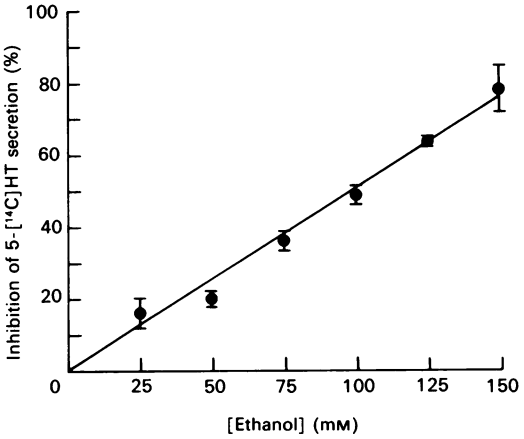


Fig. 2. Concentration-dependence of ethanol for inhibition of thrombin-induced 5-[^{14}C]HT secretion

5-[^{14}C]HT-labelled platelets were incubated for 3 min with 0.025 unit of thrombin/ml in the absence or presence of the indicated concentrations of ethanol. Each point is the mean ± S.E.M. of values from a representative experiment performed in triplicate, with individual measurements expressed as the percentage of secretion induced by thrombin alone.

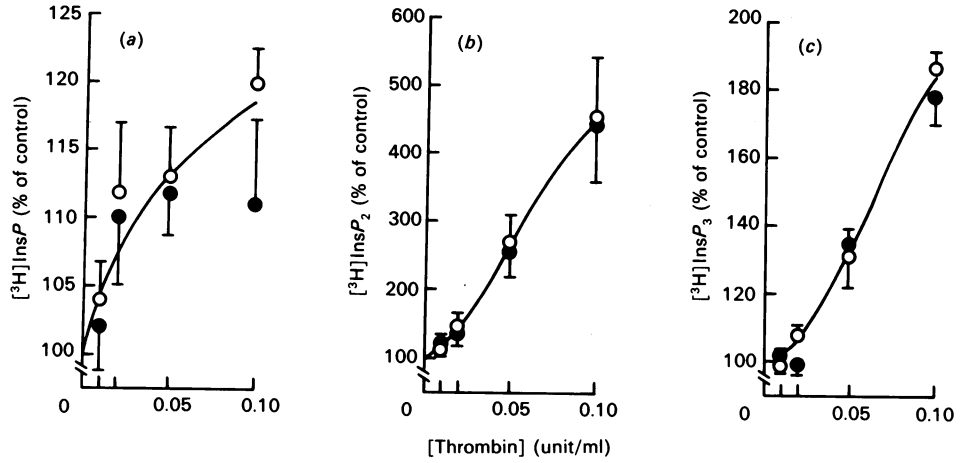


Fig. 3. Effect of ethanol on the concentration-dependence of thrombin-stimulated inositol phosphate generation in washed platelets

[^{3}H]Inositol-labelled platelets were treated for 3 min with the indicated concentrations of thrombin in the absence (○) or presence (●) of ethanol (150 mM). Inositol phosphates were extracted and separated as described in the Experimental section. Points are means ± S.E.M. of three separate experiments and are expressed as percentages of control values in the incubations without any additions. (*a*) Ins*P*; (*b*) InsP_2; (*c*) InsP_3. Mean basal levels in platelets with no additions were: [^{3}H]Ins*P*, 246 ± 40 c.p.m.; [^{3}H]InsP_2, 289 ± 51 c.p.m.; [^{3}H]InsP_3, 133 ± 12 c.p.m.

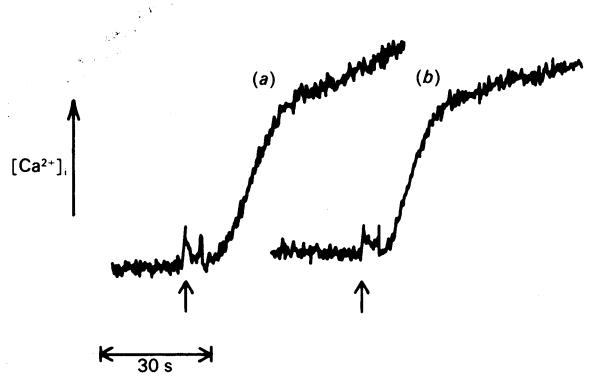


Fig. 4. Effect of ethanol on Ca^{2+} mobilization induced by thrombin

Fura2-loaded platelets were preincubated at 37 °C for 2 min before addition (arrow) of (*a*) thrombin (0.025 unit/ml) or (*b*) thrombin plus ethanol (150 mM). Measurement of fura2 fluorescence was as described in the Experimental section, and represents the 340/380 nm wavelength ratio.

Thrombin-induced mobilization of Ca^{2+} was studied in fura2-loaded platelets (Fig. 4). After addition of thrombin (0.025 unit/ml), there was a brief lag before a sharp increase in $[Ca^{2+}]_i$, which reached an inflection point within 15 s. Further measurement of fura2 fluorescence was obscured by the light-scattering effect of platelet aggregation. Preincubation of platelets with ethanol (150 mM) for 2 min minimally increased basal $[Ca^{2+}]_i$ (see [16]), but had no effect on subsequent Ca^{2+} mobilization in response to thrombin.

The activation of phospholipase C also results in DG formation. DG is then converted into PA by the action of DG kinase. Fig. 5 shows the effect of ethanol on DG and PA accumulation in platelets that were loaded simultaneously with [^{3}H]AA and [^{32}P]P_i. After addition of thrombin (0.025 unit/ml), [^{3}H]DG levels increased by 40% within 30 s, and reached maximal levels of 50% over control by 1 min. Preincubation with ethanol (150 mM) for 2 min resulted in a 30% increase in the basal levels of [^{3}H]DG. However, despite this elevation of resting levels, ethanol had no significant effect on the levels of DG after addition of thrombin. [^{32}P]PA levels increased linearly over the first 1 min after thrombin addition and reached maximal levels by 2 min. As reported previously [16], ethanol caused a small increase in basal levels of PA. However, ethanol had no effect on subsequent thrombin-induced PA formation.

Agonist-induced platelet activation is dependent, at least in part, on the stimulation of protein kinase C [21]. Fig. 6 presents the concentration-dependence of phorbol ester for 5-[^{14}C]HT secretion. A small release of 5-HT was observed in response to 10 nM-PMA, and a high concentration of PMA (50 nM) resulted in only a 9% release. In contrast with the profound effect of ethanol at these low levels of secretion in response to thrombin (see Fig. 1), ethanol had no effect on phorbol-ester-induced secretion. This observation suggests that ethanol did not interfere with the activation of protein kinase C. Therefore, in the experiment of Fig. 7, we investigated the activity of protein kinase C by measurement of ^{32}P incorporation into its major target protein, a 47 kDa protein. In ^{32}P-labelled platelets, ethanol did not interfere with the phosphorylation of the 47 kDa protein in response to thrombin (0.025–0.2 unit/ml; Fig. 7*a*) or PMA (5–50 nM; Fig. 7*b*). Similarly, ethanol did not affect the phosphorylation of myosin light chain (20 kDa) in response to these agents.

Previous studies [28] have demonstrated that the full activation of platelets is the result of the synergistic response to an increase in $[Ca^{2+}]_i$ and the activation of protein kinase C. Table 2 presents the effect of ethanol on this synergistic response. The Ca^{2+} ionophore ionomycin was used to stimulate an increase in $[Ca^{2+}]_i$. Neither ionomycin nor PMA at low concentrations significantly stimulated secretion. However, when these agents were added simultaneously there was a 31% release of 5-HT. Ethanol inhibited this response by approx. 30%.

Fig. 8 shows the effect of ethanol on the ultrastructure of control and thrombin-stimulated platelets. Resting platelets (Fig. 8*a*) are mainly discoid, and the contours are smooth. Secretory granules are evenly dispersed throughout the cytoplasm. The addition of ethanol (Fig. 8*b*) resulted in a mild shape change, as demonstrated by the conversion of many discoid platelets into spherical forms. Occasional platelets display coarse ruffling of the surface contours. Ethanol had no effect on the distribution of secretory granules. Thrombin-treated platelets (Fig. 8*c*) have all become spherical, and display numerous pseudopods. The

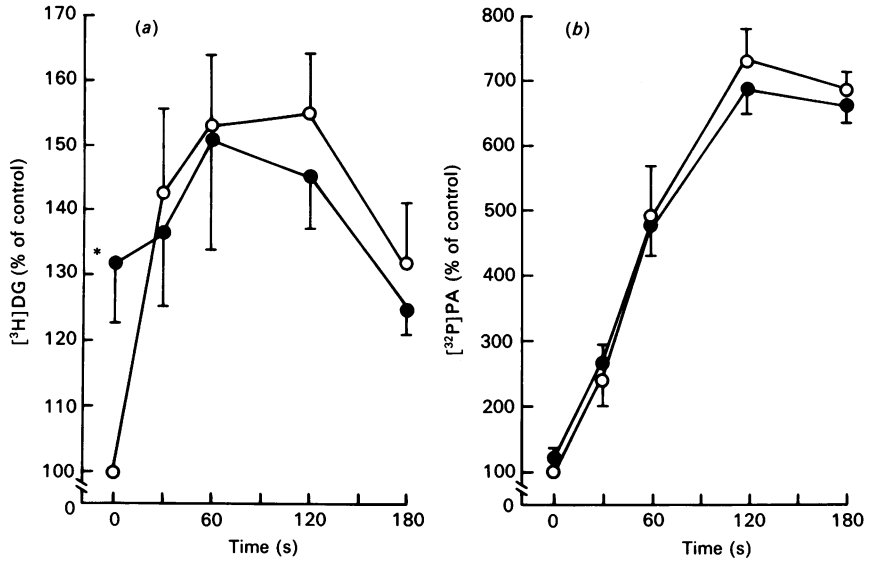


Fig. 5. Effect of ethanol on thrombin-induced DG and PA formation

Platelets were radiolabelled with both [^{3}H]AA and [^{32}P]P_i as described in the Experimental section. Samples were incubated for 2 min at 37 °C before addition of thrombin (0.05 unit/ml) in the absence (○) or presence (●) of ethanol (150 mM). Ethanol was added 2 min before the thrombin. At the indicated times, samples were taken for analysis of [^{3}H]DG (*a*) and [^{32}P]PA (*b*) as described in the Experimental section. Each point is the mean ± S.E.M. of the radioactivities combined from three or four separate experiments, with individual measurements expressed as percentages of basal levels without additions within that experiment. The mean basal levels with no additions were: [^{3}H]DG, 156 ± 26 c.p.m.; [^{32}P]PA, 185 ± 97 c.p.m. *$P < 0.02$ compared with control.

secretory granules have centralized and fused. A dense band of microfilaments surrounds the granules (Fig. 8*d*). Preincubation of platelets with ethanol (150 mM) had no effect on thrombin-induced shape change or pseudopod formation (Fig. 8*e*). However, ethanol substantially inhibited the centralization and fusion of secretory granules. Numerous bundles of microfilaments appear in the ethanol-treated platelets, but they are disorganized and do not appear to be arranged in a circular web (Fig. 8*f*). The inhibitory effect of ethanol was observed in the presence of 0.025 and 0.05 units of thrombin/ml.

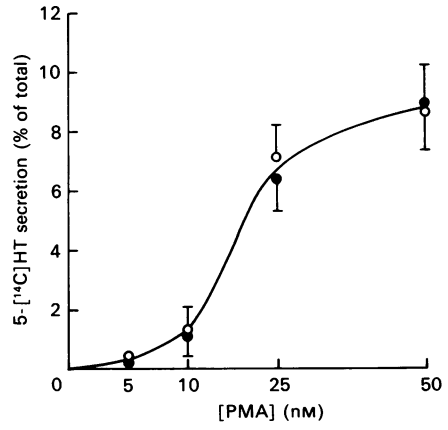


Fig. 6. Effect of ethanol on PMA-induced secretion

5-[^{14}C]HT-labelled platelets were treated with the indicated concentrations of PMA in the absence (○) or presence (●) of ethanol (150 mM). Points are means ± S.E.M. of values obtained from three separate experiments, and are expressed as the percentage of total 5-[^{14}C]HT as described in the Experimental section.

DISCUSSION

In a previous report [2], ethanol was shown to inhibit AA production in response to thrombin, apparently owing to the direct inhibition of phospholipase A_2. In the present study, the use of indomethacin allows for an analysis of the effect of ethanol on the activation of phospholipase C by thrombin, exclusive of the feedback amplification by TXA_2. In the presence of indomethacin, thrombin remains a potent stimulus for secretion, and the addition of exogenous AA does not enhance this stimulatory effect. Moreover, under these conditions, it is possible to measure the second-messenger products of phospholipase C in response to threshold doses of thrombin.

Ethanol substantially inhibited secretion in response to threshold and half-maximal concentrations of thrombin. The inhibitory effect on secretion was not overcome by the addition of exogenous AA. For several reasons, these effects of ethanol cannot be accounted for by an inhibition of phospholipase C. First, ethanol did not inhibit the formation of inositol phosphates in response to thrombin. Second, the mobilization of Ca^{2+} by a threshold dose of thrombin was unaffected by ethanol. Third, thrombin-induced formation of DG and its metabolite PA were similar in control and ethanol-treated platelets. Thus ethanol presumably acts at a site distal to phospholipase C, because it did not interfere with the primary thromboxane-independent stimulation of phospholipase C by thrombin. It is important to note that, in view of the negative effect of ethanol on second-messenger production, these data do not support an inhibitory effect of ethanol on the binding of thrombin to the plasma membrane.

That ethanol inhibits platelet activation at a locus distinct from phospholipase C or phospholipase A_2 can also be inferred from other studies. Ethanol did not interfere with the primary thromboxane-independent stimulation of phospholipase C by collagen, as measured by inositol phosphate and PA formation [2]. In studies of rabbit platelets [4], ethanol inhibited 5-[^{14}C]HT secretion by aspirin-treated platelets in response to thrombin and

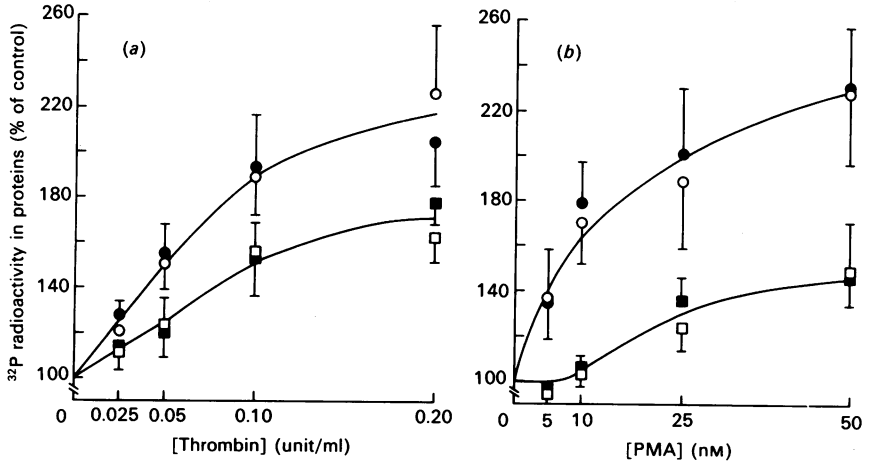


Fig. 7. Effect of ethanol on thrombin- and PMA-induced protein phosphorylation

^{32}P-labelled platelets were incubated at 37 °C for 3 min with the indicated concentrations of thrombin (*a*) or PMA (*b*), in either the absence (○, □) or the presence (●, ■) of ethanol (150 mM). At the end of 3 min, samples were quenched in SDS stop buffer and the proteins were separated by SDS/PAGE as described in the Experimental section. After autoradiography, the radioactivity in the 47 kDa (○, ●) and 20 kDa (□, ■) proteins was determined by liquid-scintillation counting. Points are means ± S.E.M. of five separate experiments and are expressed as percentages of basal values without any addition. Ethanol had no effect on basal levels of phosphorylation. Mean basal values for the 47 kDa and 20 kDa proteins were 490 ± 95 and 352 ± 81 c.p.m. respectively.

platelet-activating factor. In related studies [23], those authors also reported that ethanol inhibited slightly the formation of $InsP_3$ in rabbit platelets after addition of thrombin, under conditions in which feedback amplification by thromboxane and ADP was negligible. However, as indicated above, there is no evidence for such an inhibitory locus in human platelets.

It is generally recognized that both the mobilization of intracellular Ca^{2+} and the activation of protein kinase C are involved in eliciting various phases of the secretory response. PMA and Ca^{2+} ionophores have been used to evaluate the role of protein kinase C and Ca^{2+} in secretion, respectively. PMA provides a weak stimulus for secretion. After addition of this agent, platelets form pseudopods, but secretion does not involve granule centralization [29]. Rather, PMA appears to induce the fusion of granules, thereby leading to the formation of large cytoplasmic vacuoles [30].

Table 2. Effect of ethanol on 5-[^{14}C]HT secretion in response to PMA and ionomycin

5-[^{14}C]HT secretion was measured 3 min after addition of the indicated agents as described in the Experimental section. Data are means ± S.E.M. of three separate experiments: $*P < 0.01$ compared with PMA + ionomycin alone.

Addition	5-[^{14}C]HT secretion (% of total)
PMA (5 nM)	3 ± 1
Ionomycin (5 μM)	4 ± 2
PMA + ionomycin	31 ± 3
PMA + ionomycin + ethanol (150 mM)	20 ± 4*

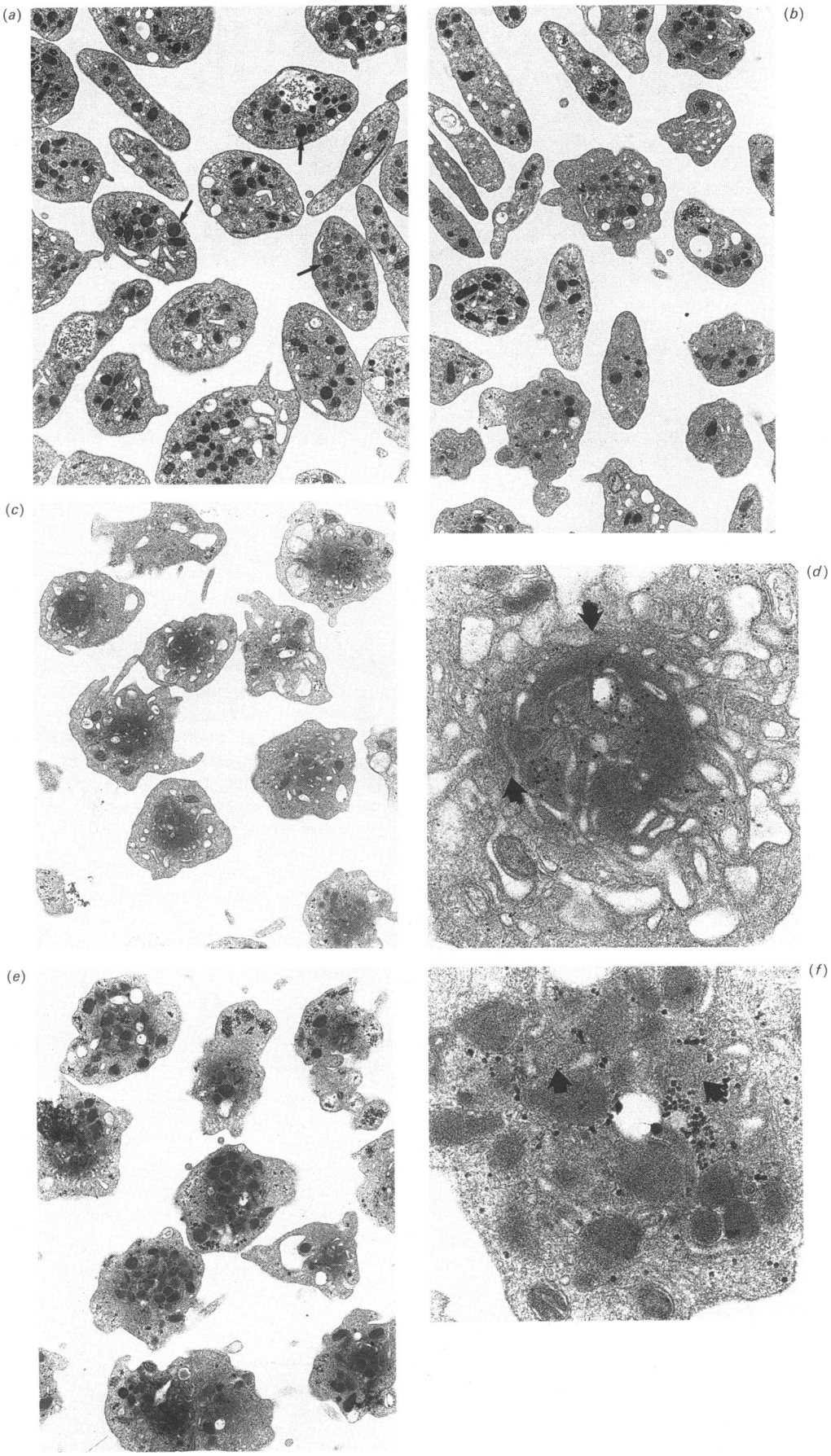
In contrast with the weak stimulation of secretion by PMA alone, the simultaneous mobilization of Ca^{2+} by the addition of ionophore leads to a strong response (Table 2). It is probable that this synergistic response underlies a mechanism by which agonists effectively induce secretion. The mechanism by which an increase in $[Ca^{2+}]_i$ stimulates secretion is only partially understood (see [22] for a review). The rise in $[Ca^{2+}]_i$ induces the formation of a complex cytoskeletal network consisting primarily of actin and myosin. Furthermore, the association of Ca^{2+} with calmodulin results in the phosphorylation of myosin light chain, a process that induces contractile activity of actomyosin. These co-ordinated processes can account for granule centralization, thereby promoting granule fusion and release.

Ethanol had no effect on the activation and function of protein kinase C by PMA. PMA-induced secretion was unaffected by ethanol. Furthermore, ethanol did not inhibit the phosphorylation of the 47 kDa protein in response to PMA or thrombin. Thus the inhibitory action of ethanol is not caused by a disruption of the protein kinase C axis of platelet signalling.

Although ethanol had no effect on PMA-induced secretion (Fig. 6), it completely inhibited secretion in response to a concentration of thrombin that caused a similar amount of phosphorylation of 47 kDa protein (see Fig. 7). This finding, in addition to those discussed above, strongly implies that ethanol inhibits a thrombin-sensitive process distinct from either phospholipase C or protein kinase C. In this regard, it has been recognized in several recent studies that such a thrombin-sensitive pathway does indeed exist. Several investigators have reported a poor correlation between thrombin-induced secretion and the activation of phospholipase C and protein phosphorylation [31,32], and in some cases secretion occurred despite the complete inhibition of these pathways [32]. In other studies of

Fig. 8 Electron microscopy of platelets

Suspensions of washed human platelets were incubated at 37 °C with (*a*) no addition, (*b*) ethanol (150 mM), (*c*) and (*d*) thrombin (0.025 unit/ml) and (*e*) and (*f*) thrombin plus ethanol. After 3 min, samples were fixed in glutaraldehyde and processed for electron microscopy as described in the Experimental section. Small arrows in (*a*) indicate secretory granules. Large arrows in (*d*) and (*f*) indicate microfilament webs. Magnification: (*a*), (*b*), (*c*) and (*e*) × 9000; (*d*) and (*f*) × 36250.


(a)
(b)
(c)
(d)
(e)
(f)

permeabilized platelets [33], the activation of phospholipase C was inhibited by the non-hydrolysable β-thio analogue of GDP, but high concentrations of thrombin still induced secretion. In a recent study [34], Watson & Hambleton used the protein kinase inhibitor staurosporine to block protein phosphorylation in platelets after stimulation with thrombin. Under these conditions, thrombin still stimulated platelet aggregation. However, when thrombin-stimulated increases in $[Ca^{2+}]_i$ were prevented, staurosporine completely inhibited aggregation. These findings suggest that Ca^{2+} may regulate a phosphorylation-independent pathway for platelet activation. These observations are also in agreement with a previous study by Rittenhouse [35] in which ionomycin caused platelet secretion and aggregation despite the full inhibition of phospholipase C and TXA_2 production.

We did not detect a primary effect of ethanol on Ca^{2+} homoeostasis. Ethanol did not interfere with phospholipase C-induced Ca^{2+} mobilization, as measured in fura2-loaded platelets, and myosin light-chain phosphorylation was also unaffected. Ethanol also did not interfere with the conversion of discoid platelets into irregular spheres and pseudopod formation, phenomena which are largely Ca^{2+}-dependent (Figs. 8*e* and 8*f*). This observation is in agreement with a previous report that ethanol had no effect on collagen-induced shape change, as measured by decreases in light-transmittance in an aggregometer [2]. Despite these findings, the present results provide evidence that ethanol does interfere with a Ca^{2+}-dependent event. First, ethanol partially inhibited the synergistic response to ionomycin and PMA. Since ethanol has no demonstratable effect on the response to PMA, it is possible that ethanol prevents those effects owing to ionomycin-induced Ca^{2+} mobilization. Second, ultrastructural studies reveal that ethanol strongly inhibits the formation of the circular contractile microfilament web and the centralization of granules, processes thought to be dependent, at least in part, on elevations of $[Ca^{2+}]_i$. It is noteworthy that ethanol also prevented thrombin-induced fusion of granules despite its inability to prevent secretion in response to phorbol ester. The reason for this discrepancy is not clear. It is possible that granule fusion in response to thrombin requires their active centralization, whereas the direct activation of protein kinase C by phorbol ester may overcome this requirement. Alternatively, ethanol may interfere with other heretofore unrecognized pathways by which Ca^{2+} and protein kinase C synergistically interact. Lastly, the present data do not exclude the possibility that ethanol may directly inhibit granule fusion.

In view of the observation that ethanol did not interfere with the formation of phospholipase C-related second-messenger products of Ca^{2+} mobilization, it is possible that the prevention of granule movement represents a primary mechanism by which ethanol inhibits secretion. In this respect, previous studies have suggested that ethanol can interact with Ca^{2+}-sensitive components of the cytoskeleton. Ethanol-induced alterations in cytoskeletal function have been reported in several cell types (reviewed in [36]). Most of these changes occur in the setting of chronic alcohol exposure or in association with the binding of acetaldehyde to microtubular components. Neither of these alternatives present realistic possibilities in the present study. Nevertheless, in view of the general interaction of ethanol with hydrophobic domains, ethanol itself could conceivably alter features of cytoskeletal assembly. For example, ethanol could affect the attachment of specific cytoskeletal components to the plasma membranes or other intracellular components by altering protein–lipid or protein–protein interactions, respectively. Alternatively, ethanol may affect the interaction of the individual components comprising the cytoskeleton. In support of this concept, it has been reported that ethanol inhibits the contractile activity of muscle actomyosin *in vitro* by blocking the interaction of actin and myosin and interfering with the responses of the complex to ADP [37]. The mechanism of this action of ethanol may be due to a non-competitive inhibition of Ca^{2+} binding with the troponin–tropomyosin complex [38]. Whether ethanol can similarly affect the contractile properties of platelet-associated actomyosin, a system primarily regulated by the phosphorylation of myosin, remains to be tested. Further studies are required to determine whether the prevention of granule centralization by ethanol represents a primary effect, or, alternatively, is the result of an interruption of heretofore unrecognized pathways of thrombin stimulation.

We have previously reported that the addition of ethanol to suspensions of stirred human platelets results in a weak stimulation of phospholipase C [16]. The elevation of basal DG levels by ethanol is likely to be a reflection of this phenomenon (Fig. 5). This action of ethanol was also illustrated in the present report by the loss of the discoid shape of platelets, as seen by electron microscopy (Fig. 8). We have hypothesized previously that the activation of phospholipase C by ethanol, with its attendant effect on Ca^{2+} metabolism and phosphoinositide turnover, may play a role in its subsequent inhibitory effect. The present findings do not support this concept, since pretreatment of platelets with a high concentration of ethanol did not alter these biochemical parameters in response to subsequent thrombin addition. Furthermore, ethanol-induced Ca^{2+} mobilization and inositol phosphate generation is transient [15,16], and, in the present studies, these variables were similar to control incubations after the preincubation period. Nevertheless, it remains to be determined whether long-term repeated exposures to ethanol could ultimately affect these pathways. In this regard, it has been demonstrated that platelets from alcoholics are defective, and regain normal function only after abstention [6,7].

In summary, in addition to its effects on phospholipase A_2 [2], ethanol interferes with thrombin-induced platelet secretion at a locus distinct from the activation of phospholipase C and protein kinase C. Moreover, ethanol does not interfere with thrombin-induced protein phosphorylation. The current findings strongly suggest that ethanol inhibits a Ca^{2+}-linked response, particularly that associated with the syngersism with protein kinase C. The movement of granules leading to centralization and fusion may be such a locus.

This work was supported by U.S.P.H.S. Grants AA-07309 and AA-07186. We thank Mr. Steven Gross for his excellent technical assistance.

REFERENCES

1. Cowan, D. H. (1980) Semin. Hematol. **17**, 137–147
2. Rubin, R. (1989) Arch. Biochem. Biophys. **270**, 99–113
3. Fenn, C. G. & Littleton, J. M. (1982) Thromb. Haemostasis **48**, 49–53
4. Rand, M. L., Packham, M. A., Kinlough-Rathbone, R. L. & Mustard, J. F. (1988) Thromb. Haemostasis **59**, 383–387
5. Fenn, C. G. & Littleton, J. M. (1983) Alcohol. Clin. Exp. Res. **7**, 59–64
6. Mikhailidis, D. P., Jenkins, W. J., Barradas, M. A., Jeremy, J. Y. & Dandona, P. (1986) Br. Med. J. **293**, 715–718
7. Desai, K., Owen, J. S., Wilson, T. & Hutton, R. A. (1986) Thromb. Haemostasis **55**, 173–177
8. Fenn, C. G. & Littleton, J. M. (1984) Thromb. Haemostasis **51**, 50–53
9. Elmer, O., Goransson, G. & Zoucas, E. (1984) Haemostasis **14**, 223–228
10. Hennekens, C. H., Willett, W., Rosner, B., Cole, D. S. & Mayrent, S. L. (1979) J. Am. Med. Assoc. **242**, 1973–1974
11. Taraschi, T. F. & Rubin, E. (1985) Lab. Invest. **52**, 120–131
12. Ponnappa, B. C., Waring, A. J., Hoek, J. B., Rottenberg, H. & Rubin, E. (1982) J. Biol. Chem. **257**, 10141–10146

13. Dorio, R. J., Hoek, J. B. & Rubin, E. (1984) J. Biol. Chem. **259**, 11430–11435
14. Suzdak, P. D., Schwartz, R. D., Skolnick, P. & Paul, S. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4071–4075
15. Hoek, J. B., Thomas, A. P., Rubin, R. & Rubin, E. (1987) J. Biol. Chem. **262**, 682–691
16. Rubin, R., Ponnappa, B. C., Thomas, A. P. & Hoek, J. B. (1988) Arch. Biochem. Biophys. **260**, 480–492
17. Rubin, R. & Hoek, J. B. (1989) Biochem. J. **254**, 147–153
18. Hoek, J. B., Taraschi, T. & Rubin, E. (1988) Semin. Liver Dis. **8**, 36–46
19. Tabakoff, B., Hoffman, P. & McLaughin, A. (1988) Semin. Liver Dis. **8**, 26–35
20. Marcus, A. J. (1987) in Hemostasis and Thrombosis (Colman, R. W., Hirsh, J., Marder, V. J. & Salzman, E. W., eds.), pp. 676–688, Lippincott, Philadelphia
21. Fenn, G. C., Caberos, L. P. & Littleton, J. M. (1985) Alcohol **2**, 177–182
22. Siess, W. (1989) Physiol. Rev. **69**, 58–178
23. Rand, M. L., Vickers, J. D., Kinlough-Rathbone, R. L., Packham, M. A. & Mustard, J. F. (1988) Biochem. J. **251**, 279–284
24. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
26. Mills, D. C. B., Figures, W. R., Scearce, L. M., Stewart, J. G., Colman, R. F. & Colman R. W. (1985) J. Biol. Chem. **260**, 8078–8083
27. Howanitz, J. H., Howanitz, P. J. & Henry, J. B. (1979) in Clinical Diagnosis and Management by Laboratory Methods (Henry, J. B., ed.), pp. 505–508, W. B. Saunders, Philadelphia
28. Rink, T. J. & Hallam, T. J. (1984) Trends Biol. Sci. **7**, 215–219
29. Carroll, R. C., Butler, R. G., Morris, P. A. & Gerrard, J. M. (1982) Cell **30**, 385–393
30. Gerrard, J. M., Beattie, L. L., Park, J., Israels, S. J., McNicol, A., Lint, K. & Cragoe, J. (1989) Blood **74**, 2405–2413
31. Haslam, R. J. & Davidson, M. M. L. (1984) Biochem. J. **222**, 351–361
32. Lapetina, E. G. & Siegel, F. L. (1983) J. Biol. Chem. **258**, 7241–7244
33. Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) J. Biol. Chem. **261**, 16838–16847
34. Watson, S. P. & Hambleton, S. (1989) Biochem. J. **258**, 479–485
35. Rittenhouse, S. (1984) Biochem. J. **222**, 103–110
36. Tuma, D. J. & Sorrell, M. F. (1988) Semin. Liver Dis. **8**, 69–80
37. Puszkin, S. & Rubin, E. (1975) Science **188**, 1319–1320
38. Puszkin, S. & Rubin, E. (1976) Arch. Biochem. Biophys. **177**, 574–584

Received 11 January 1990/26 March 1990; accepted 4 April 1990