Biochemical Pharmacology, Vol. 32, No. 14, pp. 2211–2216, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00
Pergamon Press Ltd.

INTERACTIONS OF ETHANOL WITH IONOPHORE A23187 IN HUMAN PLATELETS AND ERYTHROCYTES AND IN RAT BRAIN SLICES

LOUISA PATRIKIOU-CABEROS, MARINA A. LYNCH, CHRISTINE LEROY, G. CHRISTOPHER FENN and JOHN M. LITTLETON*

Department of Pharmacology, King's College, London WC2R 2LS, U.K.

(*Received* 6 *October* 1982; *accepted* 10 *February* 1983)

Abstract—The aggregation of gel-filtered human platelets induced by A23187 is very sensitive to inhibition by ethanol. Similarly when platelets preloaded with [^{3}H]5-hydroxytryptamine ([^{3}H]5HT) are studied in a superfusion system under conditions where aggregation is likely (high platelet density, presence of Ca^{2+}) the rate of release of [^{3}H]5HT induced by A23187 is reduced by the presence of ethanol. However when platelet aggregation is less likely (low platelet density, absence of Ca^{2+}) ethanol does not reduce the rate of [^{3}H]5HT efflux induced by A23187 in superfused platelets. In addition, in contrast to the effects of ethanol on platelet aggregation, the transformation of human red cells to echinocytes induced by A23187 is accelerated by the presence of ethanol. Similarly the increased efflux of ^{3}H from superfused rat striatal slices preloaded with [^{3}H]dopamine which is produced by A23187 is potentiated by ethanol. It is concluded that the inhibitory effect of ethanol on the action of A23187 may be confined to platelet aggregation. This may be because the mechanisms of action of either A23187 or ethanol on platelet aggregation differ from those on other cell functions.

The presence of ethanol *in vitro* has been shown to inhibit the Ca^{2+}-dependent release of neurotransmitters [1, 2] and it has been suggested that this is a consequence of a reduction by ethanol in the intracellular free Ca^{2+} concentration, either by increased binding of Ca^{2+} to membranes at the synapse [3, 4] or by decreased entry of Ca^{2+} into the nerve terminal [5]. Ethanol also inhibits platelet aggregation [6], probably by a mechanism involving inhibition of release of platelet contents [6, 7]. The aggregating agent which is most inhibited by ethanol is the Ca^{2+} ionophore A23187 [7] and ethanol also inhibits the release of platelet contents produced by A23187 when this is studied in platelet suspensions [7]. The release of platelet contents by A23187 is thought to be the result of raised intracellular Ca^{2+} concentrations caused by translocation of Ca^{2+} into the cytosol from intracellular stores by the ionophore [8]. Since ethanol does not inhibit Ca^{2+} translocation caused by another ionophore [5] and since the increased membrane disorder induced by ethanol [9] should induce a small potentiation of ionophore activity [10] these results suggest that ethanol inhibits the platelet release reaction at a step subsequent to the raising of intracellular Ca^{2+} concentrations.

This raises the possibility that inhibition by ethanol of the Ca^{2+}-dependent release of neurotransmitters is also at a step subsequent to the raising of intracellular free Ca^{2+} concentrations by membrane depolarisation. If this is so, then inhibition by ethanol of Ca^{2+} entry is likely to be of lesser importance. However, other interpretations of the experiments on platelets are possible. For example it has recently been shown that, in some circumstances, A23187-induced release of platelet contents is largely consequent on A23187-induced aggregation of platelets and that the mechanisms of the two phenomena differ [11]. Thus ethanol, by inhibiting the platelet aggregation induced by the ionophore might only secondarily inhibit the release of platelet contents. Our original experiments [7] on the inhibition by ethanol of release of platelet contents induced by A23187 were performed in unstirred suspensions of platelets in low-Ca^{2+} media. Although there is no overt evidence of platelet aggregation in such experiments this cannot be discounted as contributory to the release produced. We therefore decided to reinvestigate the interaction between ethanol and A23187 on release of platelet contents in a platelet superfusion system in which platelet : platelet contact (i.e. aggregation) might be better controlled.

Another possibility is that ethanol has some direct inhibitory effect on Ca^{2+} translocation produced by A23187, but not that by other Ca^{2+} ionophores. This would be of some importance because ethanol is frequently used as the vehicle for A23187 in biological experiments. To test this possibility we required a simpler system than the platelet and so used the A23187-induced transformation of erythrocytes to echinocytes [12–14] which is highly dependent on external Ca^{2+} concentration and is probably mediated directly by the increase in intracellular Ca^{2+} produced by incorporation of A23187 into the plasma membrane.

Lastly we considered the effect of ethanol on neurotransmitter release induced by the ionophore A23187. An interaction here might give more information on the way in which ethanol inhibits neurotransmitter release induced by more physiological means [1, 2] and could also elucidate similarities or differences between the release of intracellular con-

* To whom correspondence should be addressed.

tents by platelets, erythrocytes and neurones. By comparing the interactions between ethanol and ionophore A23187 in these three cell types we hoped to learn more about the mechanism of action of both drugs.

MATERIALS AND METHODS

Human platelets. Human blood was collected as described previously [7] from healthy volunteers into acid citrate : dextrose anticoagulant. Preparation of platelet-rich plasma with subsequent centrifugation onto bovine serum albumin and filtration on Sepharose CL2B were all as described previously [7]. The elution buffer and the medium used for suspension of gel-filtered platelets was Ca^{2+}-free Tyrode's solution (pH 7.3). The platelet count was adjusted to $3–6 \times 10^8 \cdot ml^{-1}$ before experimentation.

Aggregation of platelets in gel-filtered platelet suspensions was studied turbidimetrically in a Payton Dual Channel Aggregation Module. All experiments utilized a 500 μl cuvette at 37° with stirring at 1000 rpm. Ethanol when added to the cuvette to produce final concentrations from 12.5 to 100 mM was used as a 10 M solution in saline. A23187 was used in a vehicle containing dimethylsulphoxide (DMSO) and methanol. Adding equivalent volumes of this vehicle to platelet suspensions did not affect the aggregation produced by A23187 or collagen.

The release of [^{3}H]5HT by human platelets was studied in a superfusion system. Platelet-rich plasma was incubated with [^{3}H]5HT (25 μCi/20 ml PRP) for 60 min at 21° and then centrifuged onto bovine serum albumin before aspiration and layering onto Millipore filters (0.8 μ pore diameter). Platelets loaded with [^{3}H]5HT and trapped on Millipore filters were then superfused at $2 ml \cdot min^{-1}$ with oxygenated Ca^{2+}–Mg^{2+}-free Krebs solution containing 1 mM EGTA at 37°. After 30 min superfusion, fraction collection was begun and, after a further 10 min, platelets were superfused for 7 min (S_1) with combinations of A23187 (2.5 μM), Ca^{2+} (0.5 mM) and ethanol (50 mM). These concentrations were chosen on the basis of preliminary experiments. After 50 min further superfusion with Ca^{2+}–Mg^{2+}-free Krebs solution platelets were again stimulated (S_2) to release [^{3}H]5HT by another 7-min period of superfusion with combinations of A23187 (2.5 μM) and Ca^{2+} (0.5 mM). Superfusion and collection proceeded for a further 30 min before the experiment was terminated. The radiolabel present on the filter paper at the end of an experiment was measured by solubilisation and scintillation counting. The fraction of ^{3}H released in each period of collection was estimated from this value and from the ^{3}H present in the collected superfusate.

Human red cells. Blood was taken as described previously except that 5 mM EDTA (1 : 10 volumes) was used as anticoagulant. Whole blood was centrifuged at 3000 rpm for 5 min and serum and buffy coat removed by aspiration. Red cells were washed 3 times with buffered saline (pH 7.35, 290 ± 10 mOsm) containing 0.5% albumin before resuspension at a haematocrit of 10% and storage overnight at 4°. The next day, after 60 min incubation at 37°, 100 μl of the erythrocyte suspension was incubated at 37° with 10 ml buffered isotonic saline containing 0.5% albumin and combinations of A23187 (6 μM) Ca^{2+} (2 mM) and ethanol (50 mM). These concentrations were chosen on the basis of preliminary experiments. The vehicle for A23187 in these experiments was dimethylsulphoxide alone. Addition of the vehicle to erythrocyte suspensions did not affect the rate of transformation to echinocytes. The morphology of erythrocytes in suspension was examined by light microscopy and the echinocytotic transformation quantified by counting at intervals in a Neubauer cell.

Rat corpus striatum slices. Male Sprague–Dawley rats (200–250 g) were stunned and decapitated and striata dissected from the brain on ice. Striata were chopped into 0.2-mm prisms using a McIlwain chopper and then incubated for 60 min at 37° with [^{3}H]dopamine in oxygenated Krebs solution. After centrifugation, removal of supernatant and resuspension, aliquots of the [^{3}H]dopamine-loaded slices were placed on Whatman No. 1 filter discs and superfused at $2 ml \cdot min^{-1}$ with oxygenated Krebs solution for 60 min before collection of superfusate commenced. Release of ^{3}H from the superfused slices was induced in two periods (S_1 and S_2) during which A23187 (6, 9 and 12 μM) was present in the superfusate. The vehicle for A23187 in these experiments was DMSO. This had a small enhancing effect on its own on the spontaneous release of ^{3}H but this was not significant in relation to the A23187-induced release. The presence of ethanol (50 mM) on the release of ^{3}H induced by A23187 was tested by the incorporation of ethanol into the superfusate during S_2. The fraction of ^{3}H released in each collection period was calculated in the same way as that described for the platelet superfusion studies.

Materials. All chemicals were obtained from Sigma Chemical Co. Ltd U.K. Radiochemicals were obtained from the Radiochemical Centre (Amersham, U.K.). Rats were obtained from Charles River, U.K.

RESULTS

Human platelets

The aggregation of human gel-filtered platelets by A23187 varied considerably between individuals but ionophore concentrations of 0.2–3 μM usually caused full aggregation. Ethanol inhibited platelet aggregation produced by A23187 in all individuals but there was considerable variation. A concentration of 12.5 mM ethanol was usually sufficient to produce a greater than 50% inhibition of aggregation to a concentration of A23187 which had previously produced just maximal aggregation (Fig. 1).

Platelets preloaded with [^{3}H]5HT and superfused with A23187 or Ca^{2+} or a combination of the two released an increased fraction of ^{3}H into the superfusate (Fig. 2). The fraction of ^{3}H released by A23187 (2.5 μM) in S_1 was not totally dependent on external Ca^{2+} (i.e. some increase in release was obtained with no added Ca^{2+}) but the presence of Ca^{2+} did further increase A23187-induced release. Some increase in fractional release of ^{3}H was always obtained when

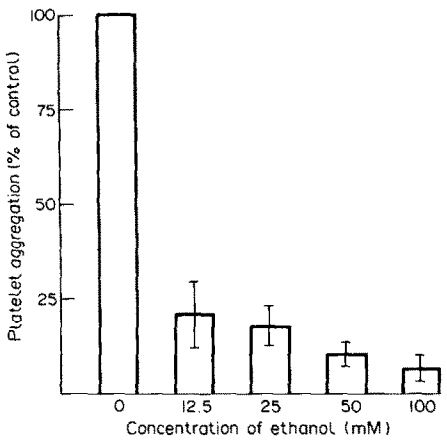

Fig. 1. Effect of ethanol on aggregation of gel-filtered human platelets induced by A23187. Results show the per cent aggregation of samples of human gel-filtered platelets in suspension. Ethanol in the concentrations shown along the baseline was added 2 min before a concentration of ionophore which had been previously shown just to produce 100% aggregation in platelets from the same individual. The values shown are the means ± S.E.M. of the percentage aggregation produced in samples from at least nine individuals.

A23187 was superfused in S_2 suggesting that irreversible aggregation and release had not occurred in all platelets during S_1.

The presence of 50 mM ethanol in the superfusate during the S_1 period of stimulation with A23187 tended to slow the rate of ^3H release when Ca^{2+} was also present (Fig. 3a) although the total fraction of ^3H released during the stimulation period was not reduced. Further examination of the data shows that the maximum rate of ^3H release in S_1 induced by the ionophore in the presence of Ca^{2+} is highly correlated with the platelet density on the filter (Fig. 4a). The presence of ethanol in the superfusate in S_1 reduces this maximum rate of release at high platelet densities but has little effect or enhances release at low platelet densities (Fig. 4b). Ethanol also had no effect on the release of ^3H produced by A23187 from [^3H]5HT loaded platelets in the absence of external Ca^{2+} (Fig. 3b). The effect of superfusion with DMSO at the concentration used as the vehicle for A23187 had no significant effect on the efflux of [^3H]5HT from platelets. Of six experiments when DMSO was superfused four showed a slight decrease and two a slight increase in the fractional release of [^3H]5HT compared with spontaneous efflux. When these results were quantified DMSO produced a mean increase in release of 0.38%. This can be compared with the 42–48% increase in [^3H]5HT release induced by superfusion with A23187.

Human red cells

The transformation of normal biconcave discocytes to echinocytes and then to spherocytes by A23187 was highly dependent on the external Ca^{2+} concentration (Fig. 5). The presence of 50 mM ethanol in the medium produced a small but significant enhancement of the rate of transformation (Fig. 5). No conditions were found under which ethanol inhibited the change produced by the ionophore.

Rat corpus striatum slice superfusion

The incorporation of A23187 in the superfusing fluid caused a concentration-dependent increase in efflux of ^3H from rat corpus striatum slices preloaded with [^3H]dopamine. A second period of incorporation (S_2) produced a very similar increase in the fraction of ^3H released. When ethanol was present in the superfusate during S_2 at a concentration of 50 mM the release induced by the ionophore was

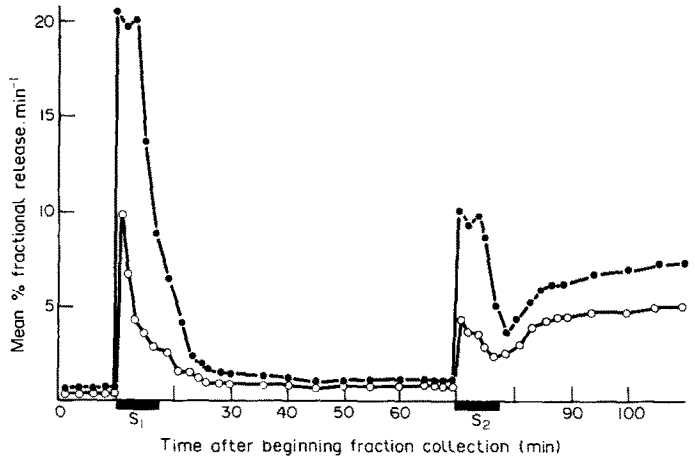

Fig. 2. Effect of A23187 on release of ^3H from superfused human platelets preloaded with [^3H]5HT. A23187 (2.5 μM) in the presence (●——●) or absence (○——○) of 0.5 mM Ca^{2+} was incorporated into the superfusate for two 7 min periods, S_1 and S_2. Values shown are the release of ^3H per minute expressed as a percentage of the total ^3H on the filter paper at the beginning of that collection period. This is termed the "mean per cent fractional release". The figure represents the mean of six experiments.

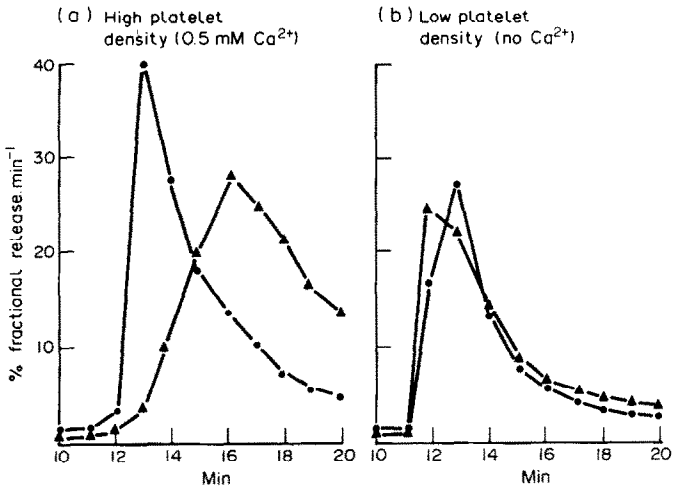

Fig. 3. Effect of ethanol on the release of ^{3}H from superfused human platelets preloaded with [^{3}H]5HT and stimulated with A23187. Results show representative results from the S_1 period of stimulation of superfusion of human platelets in which release was induced by 2.5 μM A23187 (●——●). (a) shows results in which a high density of platelets was present (as estimated by the dpm present at the beginning of fraction collection). Under these conditions the presence of 50 mM ethanol (▲——▲) reduces the rate of [^{3}H]5HT release (see also Fig. 4b) but the total release of [^{3}H]5HT in the 20 min following the first exposure to A23187 is not reduced. Thus in six separate experiments under these conditions 2.5 μM A23187 induced the release of 42.4 ± 7.8% of the total [^{3}H]5HT taken up by the platelets. When 50 mM ethanol was present the corresponding figure was 53.7 ± 7.9% (mean ± S.E.M.). These values are not significantly different. In (b) a low density of platelets was present on the filter and Ca^{2+} was absent from the superfusate. Under these conditions ethanol affects neither the rate nor the total release of [^{3}H]5HT. Thus in six experiments A23187 induced release of 47.7 ± 3.3% of [^{3}H]5HT in the absence (●——●) and 47.4 ± 1.6% of [^{3}H]5HT in the presence (▲——▲) of 50 mM ethanol.

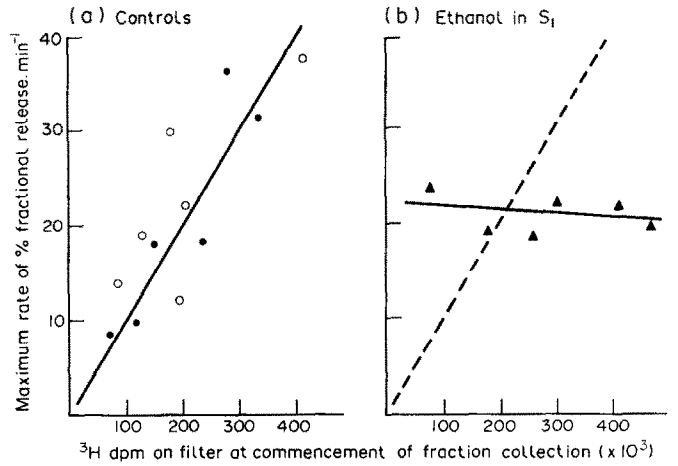

Fig. 4. Relationship between platelet density and rate of release of ^{3}H from [^{3}H]5HT preloaded platelets by A23187. (a) The results show the maximum rate of fractional release of ^{3}H induced by 2.5 μM A23187 in S_1 plotted against the dpm on the filter paper at the beginning of superfusion collection. This is taken to be an estimate of the number of viable platelets available to interact with A23187. There is a linear relationship between these variables in the control superfusion (●) when no ethanol is present (correlation coefficient 0.96) and when ethanol is present before, but not during S_1 (○) (correlation coefficient 0.72). (b) The effect of the presence of 50 mM ethanol (▲) in S_1 on the above relationship is shown. The relationship is entirely lost (correlation coefficient 0.12).

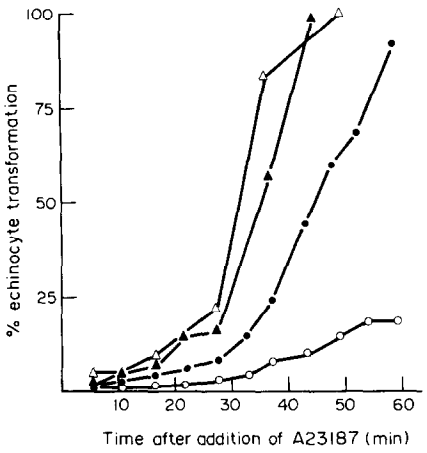

Fig. 5. Effect of ethanol on transformation of human red cells to echinocytes induced by A23187. The transformation from erythrocytes to echinocytes produced by 6 μM A23187 was assessed by light microscopy. Results shown are from one typical individual. The rate of transformation produced by A23187 in Ca^{2+}-containing buffer (●——●) is reduced by omitting Ca^{2+} from the medium (○——○) and is enhanced by addition of 50 mM ethanol either simultaneously with A23187 (▲——▲) or 1 hr before A23187 (△——△). Intermediate periods of preincubation with ethanol produced similar results. This pattern was found in all six individuals studied. However, variations in the rate of transformation of erythrocytes to echinocytes between individuals make the data difficult to compare. Nevertheless when the times to reach 50% echinocytosis are compared in a Wilcoxon signed ranks test ethanol significantly increases the rate of echinocyte production.

enhanced by 30–40% (Fig. 6). This effect was seen at all concentrations of ionophore studied.

DISCUSSION

The platelet aggregation induced by the Ca^{2+} ionophore A23187 has been reported to be very potently inhibited by ethanol [7]. Similarly the release of platelet contents induced by A23187 from platelet suspensions is also inhibited by ethanol [7]. This suggests that ethanol either inhibits the Ca^{2+} translocation induced by the ionophore or that it has an inhibitory effect on the release process by reducing the effectiveness of a raised intracellular free Ca^{2+} concentration in initiating release. A mechanism of the second kind could also explain the inhibition by ethanol of other processes thought to be triggered by raised intracellular Ca^{2+} such as Ca^{2+}-dependent neurotransmitter release [1, 2].

The results obtained in these experiments confirm the great sensitivity to inhibition by ethanol of the aggregation of human platelets when this is induced by A23187 [7]. However the interaction between ethanol and A23187 in superfused human platelets preloaded with [^{3}H]5HT suggests that ethanol does not always reduce the release of platelet contents induced by the ionophore. When the density of platelets on the superfused filter was high and when Ca^{2+} was present in the superfusate (i.e. conditions ideal for platelet aggregation to be induced by A23187) ethanol appeared to have some inhibitory effect on the release of platelet [^{3}H]5HT induced by A23187. This was manifest not as a reduction in the

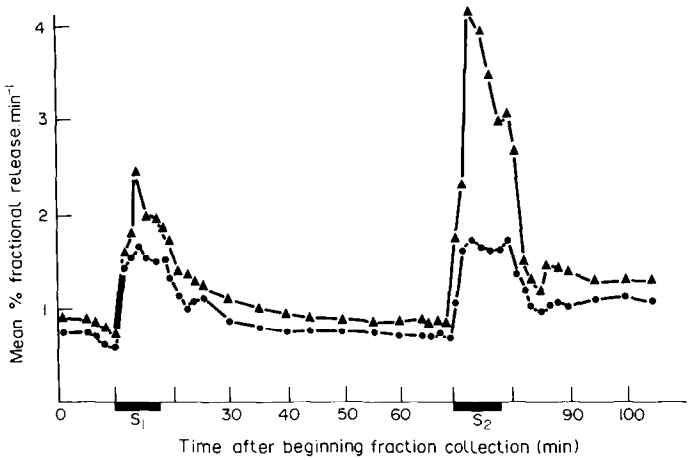

Fig. 6. Efflux of [^{3}H]dopamine from superfused slices of rat corpus striatum induced by A23187 in the presence and absence of ethanol. The results shown are the means of values obtained for the percentage fractional release of ^{3}H induced by 12 μM A23187 in S_1 and S_2 from six experiments. In the experiments denoted by the symbols ●——● no ethanol was present at any time during the superfusion. In the experiments denoted by the symbols ▲——▲ ethanol was present 40 min before, and during, S_2. Similar results were obtained with the other concentrations of A23187 investigated (6 and 9 μM). When no ethanol is present the fraction of [^{3}H]dopamine released in S_2 is very similar to that released in S_1 ($S_2/S_1 = 1.05 \pm 0.11$, mean ± S.E.M., N = 18). However when 50 mM ethanol is present during S_2 the efflux of [^{3}H]dopamine induced by A23187 is enhanced ($S_2/S_1 = 1.37 \pm 0.08$, mean ± S.E.M., N = 18). This represents a significant increase produced by ethanol at the $P < 0.05$ level in an unpaired Students test. The presence of DMSO in the superfusate at the same concentration as that present as a vehicle for A23187 produced a small enhancement of [^{3}H]dopamine release but this was insignificant compared to that produced by the ionophore plus vehicle. Increasing the concentration of the vehicle did not potentiate or inhibit the A23187-induced release.

total fraction of ^3H released during the stimulation period but rather as a reduction in the rate of release of ^3H induced by the ionophore. Prolongation of the duration of A23187-induced release by ethanol (Fig. 3a), which accounts for the similar total fraction released in the presence and absence of ethanol in the superfusate, may be a consequence of inhibition by ethanol of the platelet 5HT reuptake mechanism [15].

The maximum rate of release of platelet [^3H]5HT by A23187 in the presence of Ca^{2+} was found to be highly correlated with the density of platelets on the superfused filter. This suggests that, under conditions where aggregation is favoured, platelets release their contents more explosively in response to A23187.

Under conditions where the likelihood of platelet aggregation produced by A23187 was low (no external Ca^{2+}, low platelet density) ethanol did not inhibit the release of platelet contents induced by the ionophore.

These results suggest that the inhibition by ethanol of the A23187-induced release of platelet contents previously observed in suspensions of human platelets is a consequence of inhibition of platelet aggregation rather than a direct effect on the Ca^{2+}-mediated mechanism of release. Experiments on the echinocytotic transformation of human red cells and the release of [^3H]dopamine from slices of rat corpus striatum also show that these effects of the ionophore are not inhibited by ethanol. Ethanol in fact produces a small enhancement of the rate of red cell transformation and an increase in the [^3H]dopamine release induced by A23187. This enhancement could be related to the membrane-disordering effect of ethanol increasing ionophore mobility [10].

In conclusion ethanol has a dramatic inhibitory effect on the A23187-induced aggregation of human platelets in suspension. It does not however inhibit the release of platelet contents induced by A23187 unless this is consequent on aggregation. Ethanol also does not inhibit the echinocyte transformation of human red cells induced by A23187 and so probably does not inhibit Ca^{2+} translocation across plasma membranes produced by this ionophore. Release of the neurotransmitter dopamine by A23187 is actually enhanced by ethanol, making it unlikely that the inhibition by ethanol of more physiologically-induced transmitter release occurs at a step subsequent to Ca^{2+} entry into the nerve terminal. The most likely explanation of the differences between the interaction of ethanol and A23187 in the different cell types studied is that A23187 induces platelet aggregation by a mechanism unrelated to its Ca^{2+} ionophore effect [11]. An alternative explanation is that ethanol has some inhibitory effect on platelet aggregation which differs in its mechanism from its actions on other cells.

Acknowledgements—This research was supported by grants from the British Heart Foundation, the Medical Research Council, the Medical Council on Alcoholism and the Brewers Society.

REFERENCES

1. F. J. Carmichael and Y. Israel, *J. Pharmac. exp. Ther.* **193**, 824 (1975).
2. H. Kalant and W. Grose, *J. Pharmac. exp. Ther.* **158**, 386 (1967).
3. E. K. Michaelis and S. L. Myers, *Biochem. Pharmac.* **28**, 2081 (1979).
4. D. H. Ross, *Alcohol Tolerance and Dependence* (Eds. H. Rigter and J. C. Crabbe), p. 227. Elsevier, Amsterdam (1980).
5. R. A. Harris and W. F. Hood, *J. Pharmac. exp. Ther.* **213**, 562 (1980).
6. D. Haut and M. Cowan, *Am. J. Med.* **56**, 22 (1974).
7. G. C. Fenn and J. M. Littleton, *Thromb. Haemostas.* **48**, 49 (1982).
8. R. D. Feinman and T. C. Detwiler, *Nature, Lond.* **249**, 172 (1974).
9. J. H. Chin and D. B. Goldstein, *Molec. Pharmac.* **13**, 435 (1977).
10. M. Deleers, E. Couturier and W. J. Malaisse, *Cell Calcium* **2**, 159 (1981).
11. H. Holmsen and C. A. Dangelmaier, *J. biol. Chem.* **256**, 10449 (1981).
12. J. G. White, *Am. J. Path.* **77**, 507 (1974).
13. J. F. Kuettner, K. L. Dreher, G. H. R. Rao, J. W. Eaton, P. L. Blackshear and J. G. White. *Am. J. Path.* **88**, 81 (1977).
14. D. Allan and R. H. Michell, *Nature, Lond.* **258**, 348 (1975).
15. O. Lingjaerde, *Acta pharmac. tox.* **45**, 394 (1979).