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EFFECT OF THREE CALCIUM ANTAGONISTS ON PLATELET SECRETION AND METABOLISM*

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Abstract—Three compounds, 8-(*N,N*-diethylamino-octyl 3,4,5-trimethoxybenzoate, HCl (TMB-8), 2-propyl-3-dimethylamino-5,6-methylenedioxyindene HCl (2-PIA), and chlortetracycline, were investigated to determine whether their effects on washed human platelets were compatible with a suggested role as calcium antagonists. TMB-8 had little effect on levels of metabolic ATP and IMP, whereas chlortetracycline caused a decrease in metabolic ATP and an increase in IMP; both compounds inhibited thrombin-induced secretion and changed the platelets to spheres. At concentrations up to 0.5 mM, 2-PIA caused a decrease in ATP and an increase in IMP, an induction of secretion, and a centralization of electron-dense material in the platelets, all changes which suggest induction of secretion. The ultrastructural, functional and metabolic effects of TMB-8 and the type of interference by the drug with the effects of thrombin and 2-PIA on the same variables suggest that TMB-8 is mainly a membrane-active drug. Chlortetracycline on the other hand caused changes in platelet metabolism, ultrastructure, and function which, at least partly, indicate an effect on intracellular mechanisms. Both TMB-8 and 2-PIA, and to lesser degree chlortetracycline, caused loss of cytoplasmic nucleotides from the platelets.

The secretion of various compounds from platelet storage granules has been assumed to be controlled by movements of intracellular calcium [1]. The relative impermeability of the platelet membrane to external calcium, however, has been made impossible the direct study of calcium movement through ^{45}Ca-labeling of the intracellular calcium pool. In an attempt to circumvent this problem, calcium ionophores and compounds known to affect processes thought to be calcium regulated in other systems have been used for platelet studies. For example, Ca^{2+}-ionophores are very effective inducers of platelet secretion [2–4], and a Ca^{2+}=antagonistic effect of TMB-8 [8-*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate HCl] has been demonstrated in platelets. TMB-8 has been shown to reduce secretion and aggregation induced by thrombin, ionophores, and ADP, with the inhibitory effect of TMB-8 on ionophore-induced secretion being counteracted by addition of Ca^{2+} to the external medium [5]. 2-Propyl - 3 - dimethylamino - 5,6 - methylenedioxyindene HCl (2-PIA) has not been applied previously to blood platelets, but it has been defined as a calcium antagonist in smooth and skeletal muscle [6]. Chlortetracycline (CTC) has not been used as a calcium antagonist in muscle, although its ability to complex Ca^{2+} in a non-polar medium would suggest such a use.

TMB-8 has been shown to inhibit contraction of rabbit aortic strip, Ca^{2+} exchange in resting guinea pig ileum, and caffeine-induced Ca^{2+} efflux from rabbit skeletal muscle sarcoplasmic reticulum at 5×10^{-6} to 6.5×10^{-5} M concentrations [7, 8]. At concentrations above 5×10^{-5} M, 2-PIA inhibits: (1) the spasmogenic actions of PGE_2, $PGF_2\alpha$, oxytocin, $BaCl_2$, acetylcholine, and ergonovine maleate on estrogen-treated rat uterus, (2) the contractions of rat and guinea pig ileum induced by acetylcholine and histamine, respectively, and (3) the carbachol-induced Ca^{2+}-dependent secretion of catecholamine from perfused bovine adrenal medulla without affecting the Ca^{2+}-independent secretion induced by acetaldehyde [6, 9].

CTC apparently can be used in two ways, as an indicator of calcium translocation [4, 10–12] and also as an antagonist of calcium. CTC at 5–50 μM concentrations has been used as an indicator of Ca^{2+} translocation from a nonpolar to a polar medium because the intensity of fluorescence of the CTC–Ca complex decreases 100-fold. This property prompted the use of the drug as a probe for association/disassociation of Ca^{2+} with cell membranes [10–12]. In addition, CTC has been shown to reduce platelet secretion and aggregation induced by thrombin, ionophores, and ADP [13]. Thus, CTC may affect the process it is supposed to monitor.

* Results presented here have been presented previously at the following meetings: Annual FASEB meeting 1978 [Abstr. No. 1032, *Fedn Proc.* **37**, 407 (1978)], XIth Int. Cong. Biochem., Toronto, Canada, 1979 (Abstr. No. 05-3-R61), and VIIth Int. Cong. Thromb. Haemostas., London, UK, 1979 [Abstr. *Thromb. Haemostas.* **42**, 216 (1979)].

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The present study was designed to investigate the effects of TMB-8, 2-PIA, and CTC on platelet function, structure, and metabolism. This is necessary before we can evaluate which of their actions can be attributed to their antagonism of Ca and the value of the drugs as tools for investigating the role of calcium in platelet physiology.

MATERIALS AND METHODS

[^{14}C]Adenine (u) (281 mCi/mmole) was obtained from Amersham/Searle Arlington Heights, IL; [^{3}H]serotonin (4.3 Ci/mmole) from the New England Nuclear Corp., Boston, MA; and 2,5-diphenyloxazole (PPO), bis[2(5-phenyloxazolyl)]-benzene (POPOP), and chlortetracycline HCl from the Sigma Chemical Co., St. Louis, MO. The following compounds were gifts: imipramine from the Ciba–Geigy Corp., Summit, NJ, and 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxy-benzoate HCl from Dr. Richard Feinman, Department of Biochemistry, Downstate Medical Center, SUNY, Brooklyn, NY. 2-Propyl-3-dimethylamino-5,6-methylenedioxyindene was synthesized as described previously [14].

Blood was collected from healthy donors into 0.07 volume of 0.077 M EDTA, pH 7.4. The platelets were separated and washed twice with saline containing 0.1 mM EDTA, pH 7.4 [15].

The platelet-rich plasma that was prepared by a 15-min centrifugation at 250 *g* at room temperature was incubated with ^{3}H-labeled serotonin-binoxalate (1 μCi/ml) and ^{14}C-labeled adenine (250 nCi/ml) by shaking for 20 min at 37° in a Dubnoff metabolic incubator (75 strokes/min).

Imipramine (12.5 μM) was present during incubation to prevent reuptake of serotonin [16]. Tritium was determined in the total incubate, after stopping the experiment by cooling in an ice-bath, and in the supernatant fraction after centrifugation for 20 min at 900 *g*. Samples (25 μl) were added directly to a scintillation mixture consisting of toluene with a PPO and POPOP [2] mixture with an equal volume of Triton X-100. The counts in the ^{3}H-channel were corrected for spill over from ^{14}C. [^{14}C]Adenine metabolites were determined in total incubate and supernatant fraction by paper strip electrophoresis, according to the method of Holmsen and Weiss [17], and were counted in toluene with PPO and POPOP [2]. All radioactive determinations were done in a Beckman LS330 scintillation counter.

For electron microscopy the platelets were initially fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 30 min, followed by cold fixation for 1–2 hr. The platelets were then fixed in cold 1% osmium tetroxide for 30 min before washing and dehydration with ethanol and embedding in Epon 812. Sections were stained with uranyl acetate followed by lead citrate.

RESULTS

Dose–response of platelets to TMB-8, CTC, and 2-PIA. The dose–response curves of platelets to TMB-8 alone and to TMB-8 plus 1.25 units of thrombin are shown in Fig. 1. A 3-min incubation with TMB-8 alone caused only a modest drop in metabolic

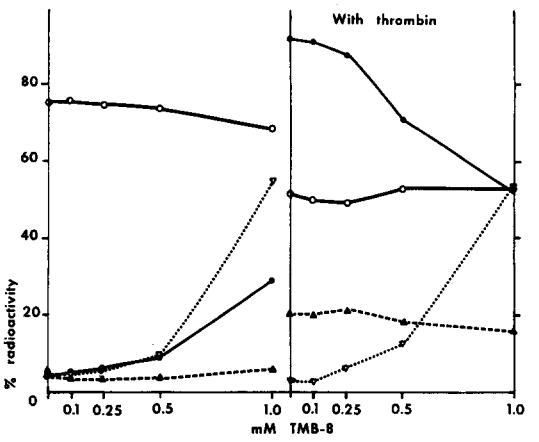

Fig. 1. Effects of various concentrations of TMB-8 on platelets in the presence and absence of thrombin. Washed platelets were suspended in 4 ml of a suspending medium containing 0.13 M NaCl, 1.2 mM EDTA–HCl (pH 7.4), 25 mM Tris–HCl (pH 7.4), and 12.5 μM imipramine in 25 ml Erlenmeyer flasks. The platelet suspension was kept in an ice-bath before use. Experiments were performed by shaking in a Dubnoff metabolic incubator at about 80 strokes/min. TMB-8 was added as a 40 mM solution in saline and was present from the beginning of the incubation as were the other added compounds. Thrombin (1.25 units/ml) was added in half the experiments. Incubation time in the present set of experiments was 3 min. Incubation was stopped by cooling in an ice-bath; thereafter a sample was removed for electrophoresis measurement of adenine nucleotides, and for determination of [^{3}H]serotonin in the total incubate. The platelets were removed by centrifugation at 900 *g* for 20 min at 5°, and samples were taken from the supernatant fraction for determination of extracellular serotonin and [^{14}C]nucleotides. Key: (○——○) [^{14}C]ATP and (▲ - - - - ▲) [^{14}C]IMP in the total incubate; (▽ - - - - ▽) [^{14}C]nucleotides and (●——●) [^{3}H]serotonin in the supernatant fraction as per cent of total [^{14}C]- and [^{3}H]-radioactivity. Mean of four experiments.

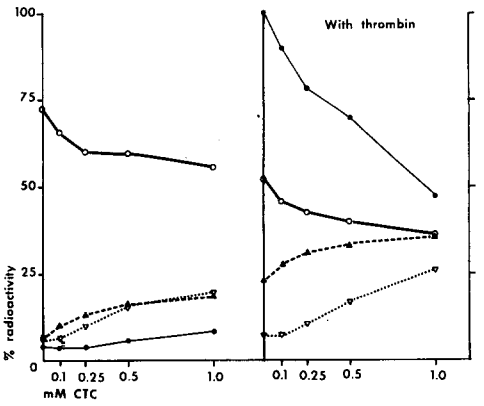

Fig. 2. Effects of various concentrations of chlortetracycline on platelets in the presence and absence of thrombin. Chlortetracycline was added as a 4 mM solution in water. To avoid hypotonic shock, 0.4 M NaCl was added before the addition of the CTC solution. Otherwise, the experiments were performed as described in the legend for Fig. 1. Mean of four experiments.

ATP, but this was accompanied by a loss of about 60 per cent of the [^{14}C]nucleotides and of about 30 per cent of the preabsorbed serotonin at 1 mM TMB-8. The loss of these compounds was minimal below 0.5 mM TMB-8.

The thrombin-induced secretion of serotonin was partially inhibited, with the extent of inhibition depending on the concentration of TMB-8. With 1 mM TMB-8, extracellular serotonin was decreased 40 per cent which, together with the increase in extracellular serotonin without thrombin, suggests a 60 per cent inhibition. The thrombin-induced increase in total IMP was slightly lower with TMB-8.

The dose–response curves of platelets to CTC alone and to CTC plus 1.25 units of thrombin per ml are shown in Fig. 2. CTC alone caused a moderate increase in the loss of [^{14}C]nucleotides and an accumulation of IMP in the total incubate over the same concentration range. There was a much smaller increase, however, in the loss of preabsorbed serotonin. The inhibition of thrombin-induced secretion of preabsorbed serotonin by CTC was in the same range as that by TMB-8, but it was initiated at a lower concentration where it coincided with a drop in metabolic ATP. The end results of CTC and thrombin addition on the changes in metabolic ATP and IMP appeared to be additive, suggesting that CTC might act as a metabolic inhibitor.

The dose–response curves of platelets to various concentrations of 2-PIA alone and to 2-PIA with 1.25 units of thrombin per ml are shown in Fig. 3.

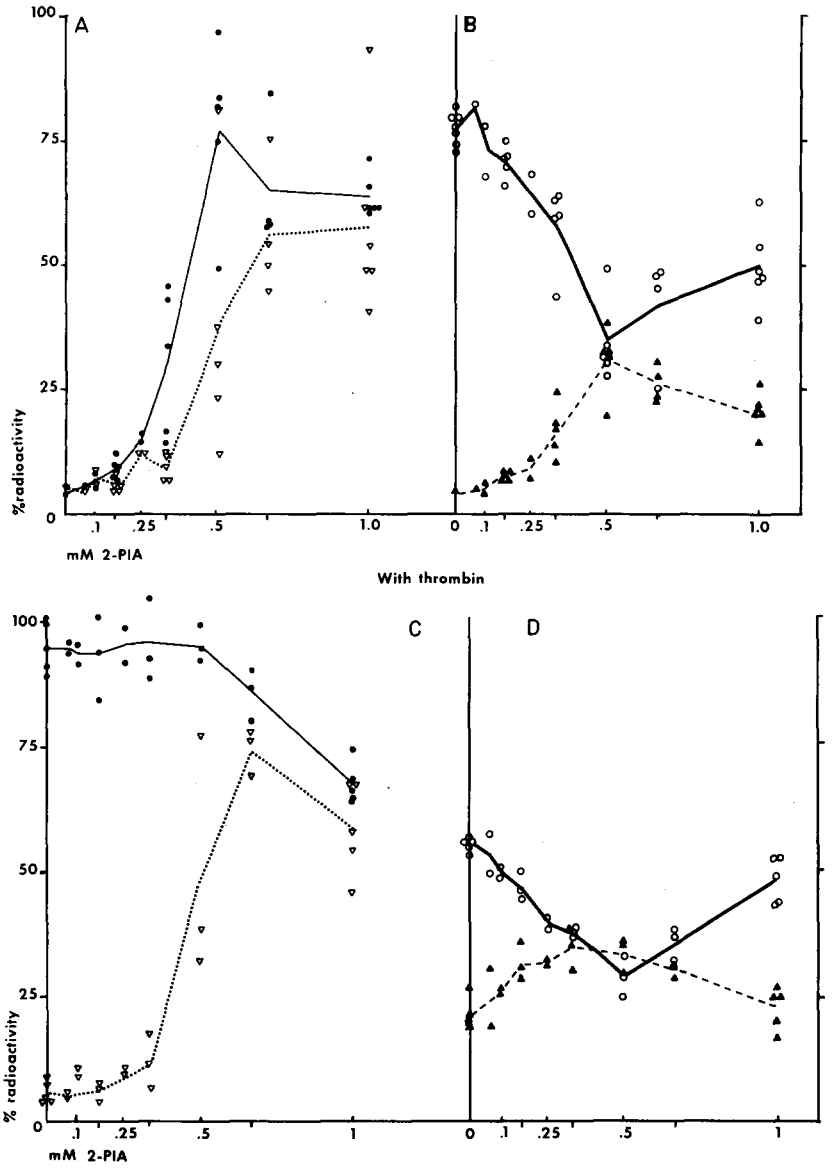

Fig. 3. Effects of various concentrations of 2-PIA on platelets in the presence and absence of thrombin. 2-PIA was added as a 40 mM solution in saline. Otherwise, the experiment was performed as described in the legend for Fig. 1. Key: (○——○) [^{14}C]ATP and (▲ - - - - ▲) [^{14}C]IMP in total incubate; (▽ - - - - ▽) [^{14}C]nucleotides and (●——●) [^{3}H]serotonin in the supernatant fraction. Composite of seven experiments.

In contrast to the other two drugs, 2-PIA alone caused a preferential loss of preabsorbed serotonin when compared with the loss of [^{14}C]nucleotides, indicating that platelet secretion was induced under these conditions. Secretion appeared to be biphasic, with the maximum occurring at about 0.5 mM 2-PIA and decreasing at 1 mM 2-PIA. The decrease would have been greater without the simultaneous increase in platelet leakage (Fig. 3A). The drug caused a dramatic biphasic drop in metabolic ATP and a corresponding increase in IMP (Fig. 3B). The thrombin-induced secretion of serotonin was partially inhibited above 0.5 mM 2-PIA. The degree of inhibition was difficult to evaluate because of the accompanying leakage (Fig. 3C). Thrombin caused a drop in metabolic ATP and an increase in IMP, which was also seen with a low 2-PIA concentration. With a high 2-PIA concentration the response of metabolic ATP and IMP was the same in the absence or presence of thrombin (Fig. 3D).

Statistical treatment of effects of 2-PIA. For five sets of data in Fig. 3, each of which contained values for 0.25, 0.5, and 1.0 mM 2-PIA, the probability values using the unpaired Student's *t*-test were determined. Statistical studies show that the biphasic effect suggested for 2-PIA (i.e. the decrease in activation when the concentration increased from 0.5 to 1.0 mM) was statistically significant for the changes in ATP and IMP ($P = 0.046$ and 0.048), while the decrease seen in release was not significant ($P = 0.16$). The reason for this may be the erratic influence of leakage. If the changes from 0.25 to 0.5 mM 2-PIA are studied for their statistical significance, it is seen that ATP, IMP, and release of serotonin changed in a highly significant manner ($P = 0.002$, 0.003 and 0.002, respectively), while the change in leakage was not significant ($P = 0.075$). These values support our conclusion that an induction of secretion took place at 0.5 mM 2-PIA, and that, at least, the metabolic changes (i.e. ATP and IMP levels) were reversed to a significant degree when the 2-PIA concentration was increased from 0.5 to 1.0 mM.

Ultrastructural changes induced by TMB-8, CTC and 2-PIA. Electron microscopic studies also showed several drug-induced changes in platelet ultrastructure: platelets that were washed with EDTA-saline (control) were no longer discoidal in shape, possessed pseudopods, and exhibited moderate centralization of granules (Fig. 4). After a 3-min exposure to CTC, the platelets were nearly spherical with smooth contours, i.e. the pseudopods had been retracted. In addition, the granules were evenly distributed (Fig. 5). After a 3-min exposure to 2-PIA, a condensed mass of electron-dense material was seen in the majority of the platelets that also showed various degrees of degranulation (Fig. 6). After a 3-min exposure to TMB-8 the platelets were almost completely spherical with smooth contours (Fig. 7). The changes were more pronounced than with CTC.

DISCUSSION

The study of the role of intracellular calcium in the regulation of cell function and metabolism has been limited by the difficulty of obtaining selective and sensitive measurements of Ca^{2+} binding and transport. In platelets, the study is further complicated by the fact that extracellular calcium does not enter the cytoplasm, and thus we cannot label the intracellular Ca^{2+}. One approach to a solution of this problem has been the use of chemical compounds that may react in some way with calcium or that may interfer with certain calcium-dependent cell functions. This approach, however, is seriously limited in that it can give only a summation of the effects of the action of the compound on all processes, whether calcium-dependent or calcium-independent. Furthermore, calcium-dependent processes may be influenced secondarily to the primary effects of calcium-independent processes.

We have presented here the effects of three compounds (TMB-8, CTC, and 2-PIA) that have been reported to influence some calcium-dependent function in cells or cell organelles (see introduction). These compounds were used alone, with thrombin, and, to a limited extent, in combination, to study platelet secretion, adenine nucleotide metabolism, plasma membrane integrity, and ultrastructure. Metabolic changes induced by thrombin (glycogenolysis, lactate production, and changes in nucleotide metabolism) are fairly well documented. Furthermore, these changes occur without loss of cytoplasmic constituents, i.e. membrane permeability to larger molecules is not altered [18].

A very significant finding of this study was that each compound alone produced changes in some aspect of adenine nucleotide metabolism, plasma membrane integrity (Figs. 1–3), and ultrastructure (Figs. 4–7) in a dose- and time-dependent manner. These changes occurred at low drug concentrations (0.1 to 1.0 mM) and short incubation (3-min) times. 2-PIA also produced considerable release of serotonin from platelets, though this might have been partly through leakage as well as secretion. The characteristics of the platelet response for each drug differed in some details, such as effective dose and kinetics and extent of response (for an overview, see Fig. 8).

There was a striking difference between the loss of cell content induced by TMB-8 and that by 2-PIA, since 2-PIA caused a preferential loss of serotonin but TMB-8 (and to a lesser degree CTC) caused a loss of nucleotides. This suggests that TMB-8 in this respect acts as membrane-active compound, similar to sulocticil,* which also causes preferential loss of cytoplasmic nucleotides at higher concentrations [19]. On the other hand, the preferential loss of granule-stored material induced by 2-PIA is taken as evidence that this compound acts as an inducer of secretion.

* Sulocticil [1(4-isopropylthiophenyl)-2-*n*-octylaminopropanol] is a vasodilating drug that causes a specific release of serotonin from platelets at low concentrations. It has a diphasic effect on red cells, reducing hemolysis at 1–6 μM concentrations and increasing membrane permeability above 20 μM. In platelets, a preferential loss of cytoplasmic over granule-stored material is observed at the higher concentrations. This is in contrast to the effect of chlorpromazine, which causes loss of granule-stored and cytoplasmic constituents in tandem [19].

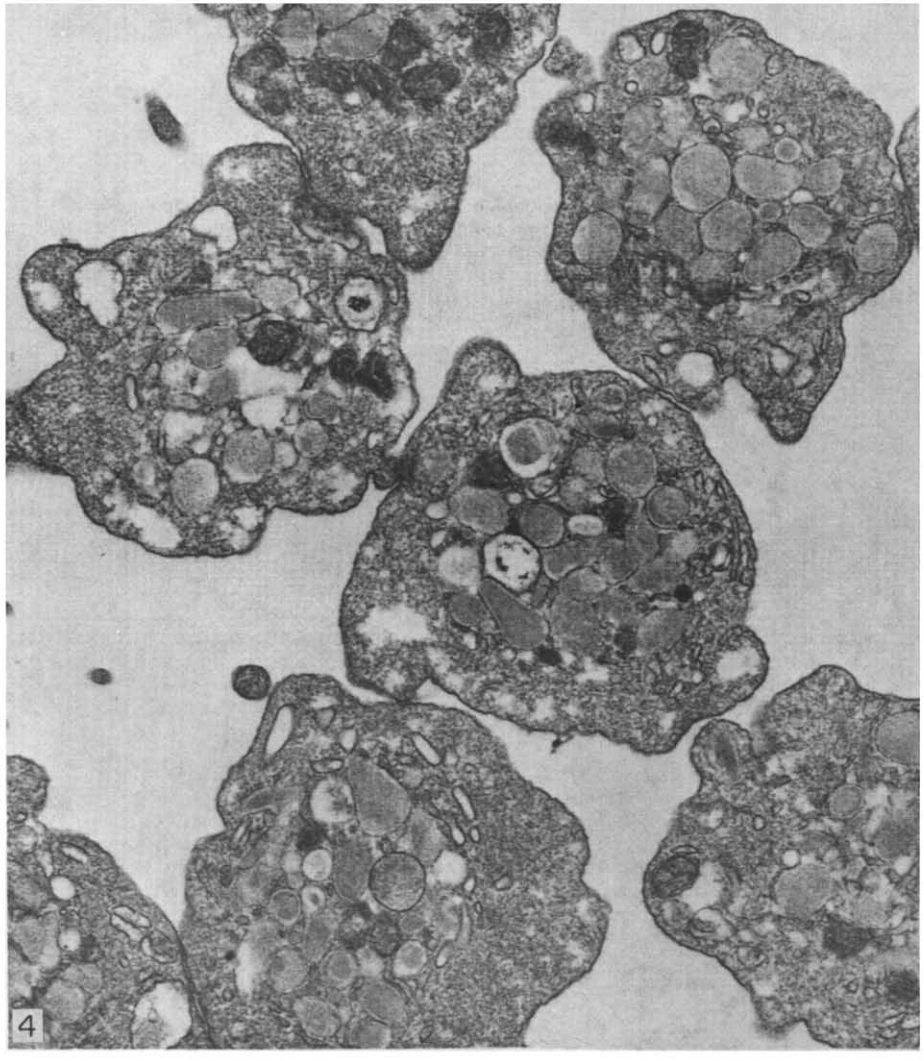

Fig. 4. Electron microsocope study of washed platelets. Platelets were incubated for 3 min without drugs (control) under the conditions outlined in the legend for Fig. 1; then 2 ml platelets were added to 2 ml of the glutaraldehyde solution at room temperature. Platelets were somewhat activated with moderate centralization of granules. Magnification: 26,600.

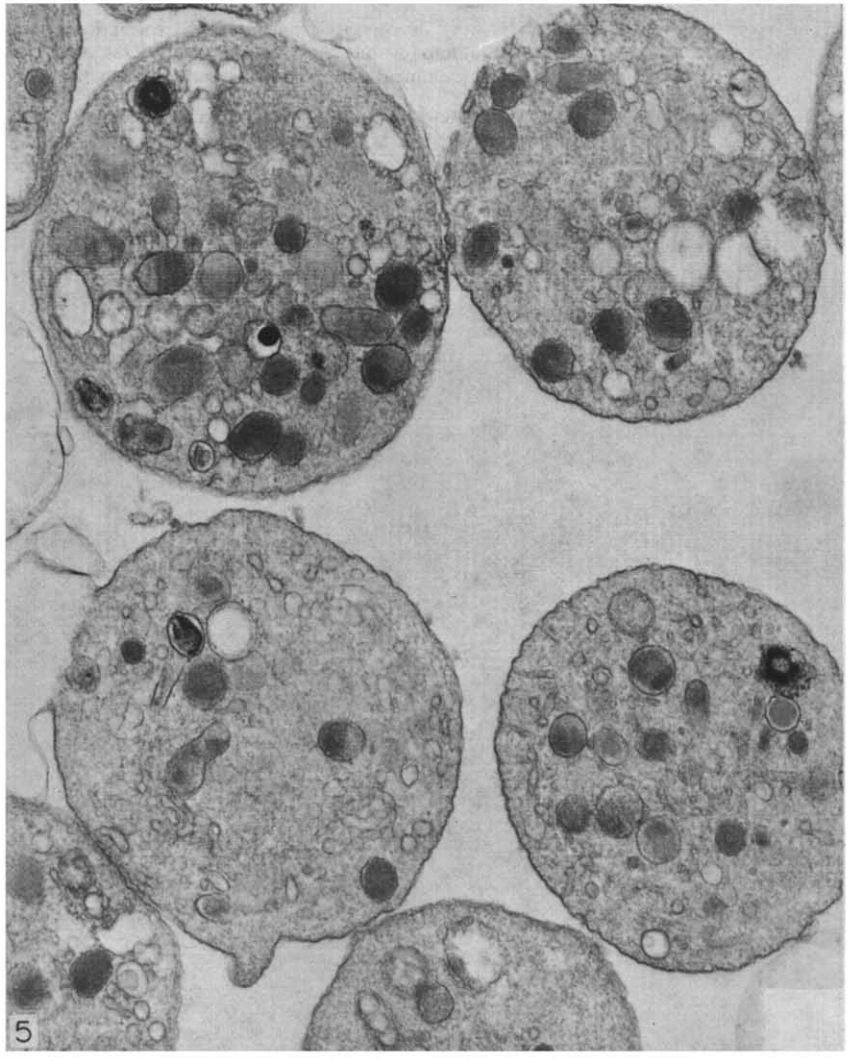

Fig. 5. Platelets from the same preparation incubated with 1 mM chlortetracycline for 3 min. Platelets were spherical with smooth contours. Granules were evenly distributed. Magnification: 26,300.

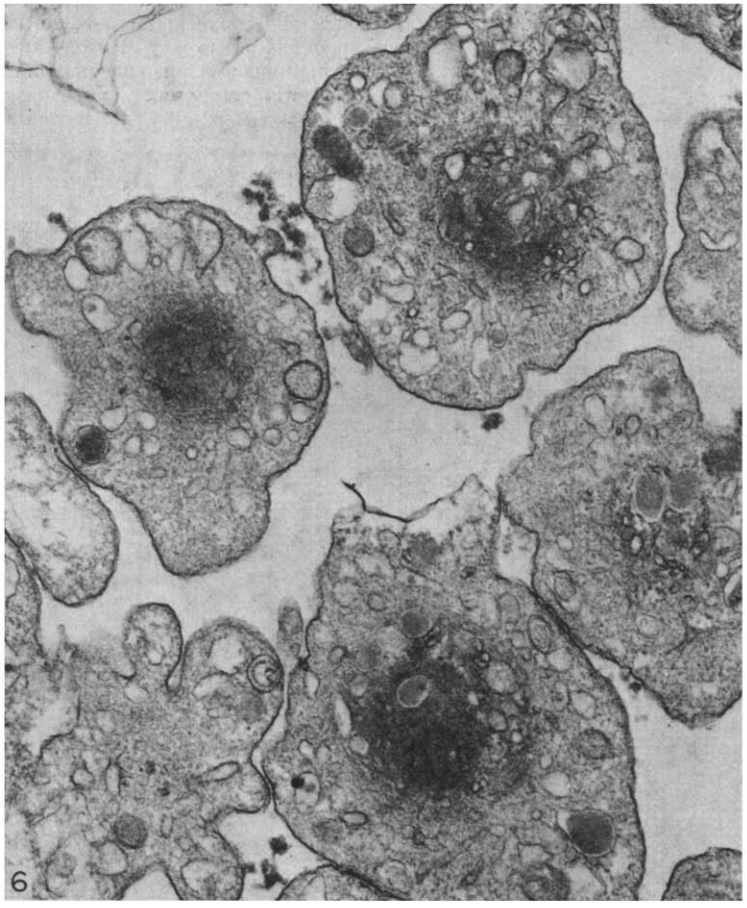

Fig. 6. Platelets from the same preparation incubated with 0.5 mM 2-PIA for 3 min. A condensed mass of electron-dense material and a different degree of degranulation were seen. Magnification: 24,100.

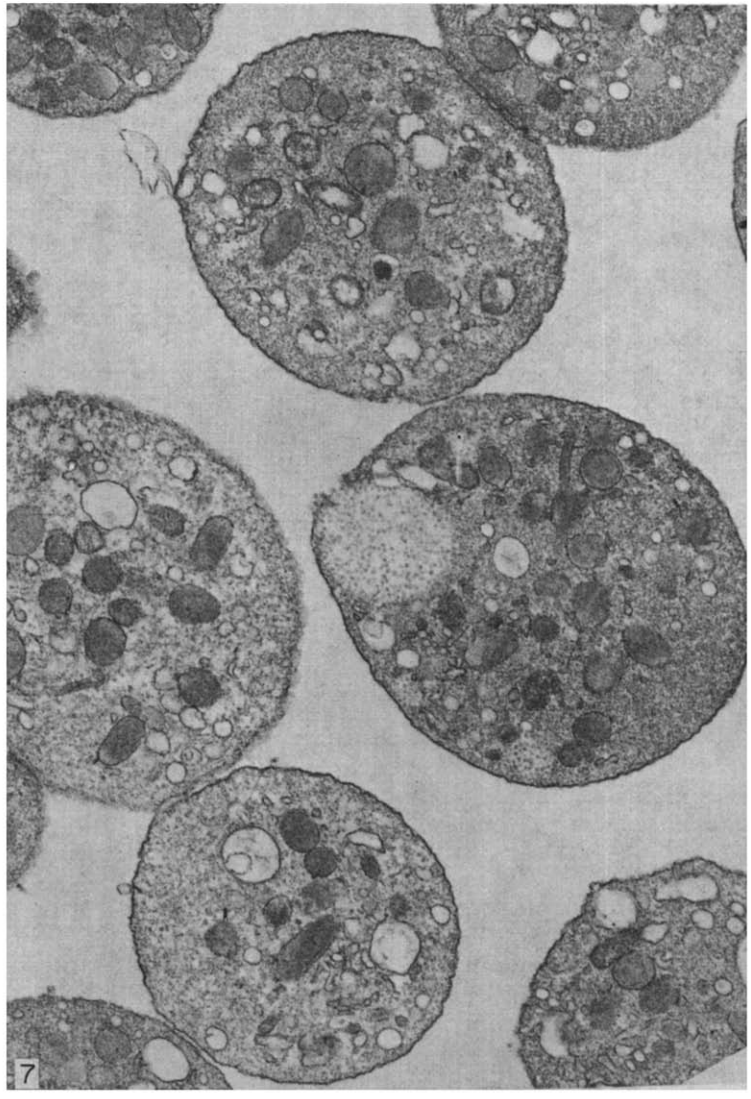

Fig. 7. Platelets from the same preparation incubated with 1 mM TMB-8 for 3 min. Platelets were almost completely spherical. Magnification: 24,600.

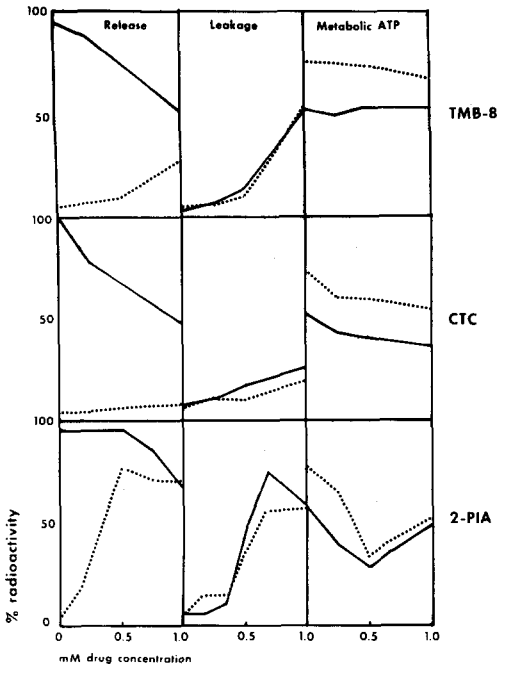

Fig. 8. Comparison between the effects of TMB-8, CTC, and 2-PIA on platelet function, structural integrity, and metabolism. Experiments were taken from Figs. 1–3. Solid lines: thrombin added. Dotted lines: no thrombin.

The metabolic effects of TMB-8 were very slight. TMB-8 caused no increase in IMP accumulation, in contrast to CTC. TMB-8, however, exerted a striking inhibition of thrombin-induced secretion, as well as greatly increased leakage. It also inhibits the thrombin-activated phospholipase A_2 activity of platelets [20], whereas the A23187-induced phospholipase activity is much less affected. This may indicate a compromised plasma membrane, suggesting that many of the actions of TMB-8 may be ascribable to a membrane-active drug that interferes with the interaction between thrombin and active sites on the platelet surface. Le Breton and Dinerstein [21] showed previously that an analog, (*N,N*-dimethylamino) hexyl-3,4,5-trimethoxybenzoate (TMB-6), inhibited thrombin- and ADP-induced platelet secretion, but not ADP-induced platelet shape change and the accompanying calcium redistribution (as measured by change in CTC fluorescence). They explained the discrepancy (that one, but not the other presumably calcium-dependent reaction was inhibited) by postulating two pools of intracellular calcium, one regulating secretion, and the other shape change. Their finding may, however, not be incompatible with our proposal that the TMB-type compounds function as membrane-active drugs.

The method by which the platelets were prepared does not seem to have influenced the activity of the drugs. The degree of leakage induced by TMB-8 and CTC and the inhibitory action of CTC on thrombin-induced secretion were at least as great with gel-filtered platelets [22] prepared and studied in the absence of EDTA and without previous cooling in an ice-bath (E. Mürer and E. Siojo, unpublished observations).

The ultrastructural studies provide evidence as to the effects of these drugs on the platelet contractile system. This system is strongly influenced by the level of free calcium in the cytoplasm. Since the microtubules were depolymerized during EDTA washing and storage in the cold, platelet shape changes could be attributed to the contractile system. Thus, the irregular shape of control platelets and the moderate centralization of granules reflected some degree of activation during handling, presumably due to liberation of intracellular calcium. After exposure to 1 mM TMB-8 or CTC for 3 min the platelets became spherical and the granules dispersed. This would be expected if the action of calcium were antagonized so that the small degree of contraction was reversed. In this respect, the two agents appeared to be "calcium antagonists". In contrast, exposure of platelets to 0.5 mM 2-PIA for 3 min caused further activation of the platelets, as indicated most strikingly by the development of centralized balls of microfilaments. These have been identified as actin [23] and have been observed in platelets activated by a number of agents [24]. Furthermore, the platelets remained irregular in shape. This is in agreement with the observation that 2-PIA induced [^{3}H]serotonin secretion (Fig. 3A).

In conclusion, it is apparent that TMB-8, 2-PIA, and CTC each affect several platelet variables. Some of the effects, such as inhibition of thrombin-induced secretion and reversal of minimal contraction of the microfilament network by TMB-8 and CTC, could depend on the antagonism of calcium. On the other hand, 2-PIA may act as a calcium antagonist by partially inhibiting thrombin-induced secretion but as an agonist by causing formation of balls of microfilaments and inducing secretion. All three have the disadvantage of causing considerable leakage of cytoplasmic constituents, i.e. all alter the plasma membrane. The effects of the drugs are dose and time dependent. Thus, the drugs must be used with an awareness of the complexity of effects and the importance of the dose and time of exposure.

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