MOLECULAR PHARMACOLOGY, 21:422–431

Effects of the Calmodulin Antagonist Trifluoperazine on Stimulus-Induced Calcium Mobilization, Aggregation, Secretion, and Protein Phosphorylation in Platelets

M. B. FEINSTEIN AND R. A. HADJIAN

Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032

Received June 11, 1981; Accepted October 29, 1981

SUMMARY

The calmodulin antagonist trifluoperazine inhibited platelet aggregation and the secretion of calcium and ATP from the dense granules and *N*-acetylglucosaminidase from lysosomes. The inhibition of aggregation and secretion occurred concurrently but could also be demonstrated independently of each other and in the absence of any effect on stimulus-induced platelet shape change. All platelet-stimulating agents tested were antagonized, i.e., thrombin, trypsin, collagen, epinephrine, A23187, melittin, thimerosal, and arachidonic acid. Previous studies [Walenga, R. W., E. E. Opas, and M. B. Feinstein, *J. Biol. Chem.* **256**:12523–12528 (1981)] showed that trifluoperazine can block thrombin-induced release of arachidonic acid from phosphatidyl choline and thromboxane formation without inhibiting breakdown of phosphatidyl inositol or the formation of phosphatidic acid. Despite a lack of effect of trifluoperazine on the metabolism of exogenous arachidonic acid, both arachidonic acid- and prostaglandin H_2-induced aggregation and secretion were antagonized. Trifluoperazine could also completely suppress thrombin-induced phosphorylation of platelet proteins, notably the M_r 20,000 myosin light-chain and an M_r 47,000 protein. The effect on the latter may be due to inhibition of the Ca^{2+}-dependent, phospholipid-activated, protein kinase C, whereas the effects on myosin phosphorylation can be attributed to inhibition of *calmodulin-dependent* myosin light-chain kinase. Similarities in the process of activation of protein kinase C and calmodulin-dependent enzymes may account for their common susceptibility to the same class of inhibitors. Using chlortetracycline as a fluorescent probe for intracellular membrane-bound calcium, it was found that stimulation of platelets releases Ca^{2+} intracellularly prior to secretion and protein phosphorylation. Trifluoperazine acted to *uncouple* activation from secretion: at low concentrations the drug inhibited secretion, although intracellular Ca^{2+} release was either not affected or actually markedly speeded up by the drug. Higher concentrations of trifluoperazine partially inhibited later stages of calcium release and almost totally blocked secretion. These data provide supporting evidence for the involvement of calmodulin in mediating certain effects of calcium in platelets, but they also suggest that other calmodulin-*independent* processes are also targets for trifluoperazine.

INTRODUCTION

Calcium serves as a second messenger or modulator linking the stimulation of cell surface receptors to intracellular control of enzymes involved in certain aspects of carbohydrate, lipid, and cyclic nucleotide metabolism. Calcium is also an important regulator of cellular functions such as excitability, membrane permeability and ion transport, contraction, motility, and exocytosis. The calcium-binding protein calmodulin serves as an intracellular receptor for Ca^{2+} and mediates the stimulation of many calcium-dependent enzymes, e.g., cyclic nucleotide phosphodiesterase, brain adenylate cyclase, red cell membrane (Ca^{2+} + Mg^{2+})-ATPase, phosphorylase *b* kinase, membrane protein kinases, myosin light-chain kinase, and phospholipase A_2 (1).

Calmodulin is present in platelets, and it is a reasonable assumption that it may mediate some of the Ca^{2+}-dependent responses of platelets to stimulation, such as aggregation, release of products stored in various secretory organelles, production of free arachidonic acid by lipolytic action on phospholipids, and activation of contractile proteins. Calmodulin is a required cofactor of platelet myosin light-chain kinase, which, like its counterpart in smooth muscle, is responsible for Ca^{2+}-dependent phosphorylation of the M_r 20,000 myosin light-chain that is necessary for actin-activated ATPase activity and force generation (2). Platelet phospholipase A_2, which is in-

This research was supported by National Institutes of Health Grant HL18937.

0026-895X/82/020422-10$02.00/0

volved in the release of arachidonic acid from certain phospholipids, has also been reported to be stimulated by calmodulin in membrane preparations (3).

The experiments reported in this paper represent an assessment of the possible involvement of calmodulin in the responses of *intact* platelets to aggregating agents. The approach has been to study the effects of trifluoperazine, a potent inhibitor of calmodulin, on various aspects of the platelet's reactions to stimulation. Trifluoperazine forms a ternary complex with calmodulin-Ca^{2+} that prevents calmodulin from activating its target enzymes (4). In this way, trifluoperazine and other inhibitors of calmodulin can inhibit smooth muscle contraction by preventing myosin light-chain phosphorylation (5). Inhibition of platelet aggregation (6–9), thrombin-induced secretion of serotonin (7), and arachidonic acid mobilization (8, 9) by trifluoperazine have recently been reported.

MATERIALS AND METHODS

Human platelet concentrates containing CPDA-1 (an anticoagulant solution comprised of citrate, phosphate, dextrose, and adenine) were purchased from the Connecticut American Red Cross Blood Center and used within 12 hr after being obtained from the donor. The platelets were kept at 20–24° on a rotator until used. For experiments involving measurements of aggregation or secretion the platelet-rich plasma was diluted as required into a buffered salt solution containing 25 mM Tris-HCl, 137 mM NaCl, 5.4 mM KCl, 1.0 mM $CaCl_2$, and 0.2% dextrose to a final pH of 7.4 or 8.0. When washed platelets were used they were prepared as previously described (10) and then resuspended finally in the solution described above. Platelet aggregation was measured photometrically in a Chronolog aggregometer. Each batch of platelets was tested prior to an experiment for its ability to produce malonyldialdehyde and for the rate and magnitude of secretion and aggregation in response to test doses of thrombin and collagen. Only platelets giving suitable control responses were used.

N-Acetylglucosaminidase secretion. Platelet suspensions containing 2.0 mg of platelet protein per milliliter were incubated at 37° or 21° at pH 7.4 or 8.0. Samples (0.5 ml) were withdrawn at intervals after addition of a stimulating agent and added to test tubes in an ice bath. After centrifugation for 30 sec in an Eppendorff centrifuge (8000 × *g*), 0.2-ml aliquots of the supernatant solution were added to 0.8 ml of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M sodium citrate (pH 4.3) and incubated with mixing for 1 hr at 37° (11). The reaction was stopped by the addition of 1.0 ml of 0.4 M glycine (pH 10.5). The absorbance of released *p*-nitrophenol was measured in a Gilford spectrophotometer at 420 nm. The molar absorbance of *p*-nitrophenol is 1.62×10^4.

Calcium secretion. Platelet suspensions (1.0 mg of platelet protein per milliliter) were incubated at 37° or 21° at pH 7.4 or 8.0. At suitable times after addition of a stimulating agent, 1.0-ml aliquots were added to tubes containing 0.15 ml of ice-cold 9% paraformaldehyde and 2 mM EDTA. After centrifugation in an Eppendorff centrifuge the supernatants were analyzed for calcium by atomic absorbance spectroscopy as previously described (10). A continuous measurement of the secretion of calcium was attained by using a calcium electrode (12). Trifluoperazine did not affect the response of the electrode to additions of Ca^{2+} standards into platelet suspensions.

Simultaneous measurement of Ca^{2+} secretion and the release of intracellular Ca^{2+}. CTC[1] fluorescence was employed as probe to measure membrane-bound calcium as previously described (12). Platelets concentrated in their own plasma (20–30 mg of platelet protein per milliliter) were incubated with 10–50 μM CTC for about 30 min at 21–22°. Samples were diluted with 1.0 ml of buffered saline solution and centrifuged for 15 sec in an Eppendorf microcentrifuge. The diluted plasma was removed by aspiration, and the platelets were resuspended in a CTC-free buffered saline solution to a concentration of about 120–380 μg of platelet protein per milliliter. The secretion of calcium and the CTC fluorescence in platelets were measured simultaneously in a Perkin Elmer MPF2 fluorometer. The sample compartment contained a cuvette into which a Ca^{2+} electrode (Radiometer F2112Ca) and an Ag/AgCl reference electrode were placed. Rapid mixing was provided by a powerful magnetic stirrer. The electrodes were connected to a Radiometer 25 pH meter whose output was led to a strip chart recorder. The electrode response was calibrated by adding known amounts of standard Ca^{2+} solutions to platelet suspensions with a Hamilton syringe through a small opening in the cover of the sample chamber of the fluorometer. The medium initially contained 50–100 μM Ca^{2+}, which enables a rapid response of the Ca^{2+} electrode. Fluorescence excitation was at 390 nm and the emission at 90° was measured at 530 nm. By using low concentrations of cells and a 430-nm emission cutoff filter, the contribution of light scattering to the fluorescence signal was kept negligible. The fluorescence method can detect changes in binding of Ca^{2+} to membranes when the free Ca^{2+} is in the micromolar range. The over-all response time of the instrument, including mixing, for both Ca^{2+} activity measurements and CTC fluorescence was such that for both signals at least 90% of maximal response was attained within 1.0 sec.

Simultaneous measurement of platelet aggregation and ATP secretion. Secretion of ATP and platelet aggregation were measured at the same time in a Lumiaggregometer (Chronolog Corporation, Havertown, Pa.) as described by Feinman *et al.* (13). Luciferin-luciferase reagent (Chrono-Lume, Chronolog Corporation) was added to platelet suspensions to detect ATP secretion by light emission with a highly sensitive photomultiplier tube. The $MgSO_4$ in this reagent brought the Mg^{2+} concentration to 2.0 mM in each assay tube. The presence of Mg^{2+} did not affect the responses to trifluoperazine when secretion was measured by the other methods described above. Aggregation was measured by light transmittance in the infrared region. Platelet suspensions (0.45 ml) were kept at 31° or 37° and stirred with a Teflon-coated magnetic stir bar at 1000 rpm.

[1] The abbreviations used are: CTC, chlortetracycline; SDS, sodium dodecyl sulfate; PGH_2, prostaglandin H_2; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; LC_{20}, myosin light-chain *M*, 20,000.

Protein phosphorylation. Washed platelets were suspended in 10 ml of medium containing 25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5.4 mM KCl, and 0.2% dextrose. The platelets were loaded with $^{32}PO_4$ for 40–60 min at a final specific activity of 0.1 mCi/ml at room temperature. Isotopic equilibrium between intracellular phosphate and ATP is attained with a 30-min pulse labeling with $^{32}PO_4$ (14). After this incubation the platelets were washed to remove extracellular radioactive phosphate and suspended at a concentration of 1 mg of platelet protein per milliliter in the buffered saline solution described above containing 1.0 mM $CaCl_2$ plus or minus trifluoperazine at either 50 μM or 100 μM. After incubation at 37° for 5 min the platelets were stimulated by addition of thrombin (1.0 unit/ml). Reactions were stopped at various times with an SDS-dithiothreitol solution yielding final concentrations of 1.1% and 50 mM, respectively, and heated immediately for 2 min at 100°. Proteins were separated by electrophoresis on 12% polyacrylamide, discontinuous pH, 1% SDS gels. The molecular weight standards were as follows: phosphorylase *b*, M_r 94,000; bovine serum albumin, M_r 68,000; glutamic dehydrogenase, M_r 53,000; aldolase, M_r 40,000; lactic dehydrogenase, M_r 36,000; soybean trypsin inhibitor, M_r 21,000; and cytochrome *c*, M_r 11,700. The incorporation of $^{32}PO_4$ into specific proteins was analyzed by autoradiography of dried slab gel plates on Kodak X-Omat R film and densitometry of the developed X-ray film (15).

The sources of materials used in this study were as follows: thrombin, 3000 units/mg of protein (Sigma Chemical Company, St. Louis, Mo., or Calbiochem, San Diego, Calif.), trypsin (Sigma Chemical Company), luciferin-luciferase reagent (Chronolog or Calbiochem), arachidonic acid (Sigma Chemical Company or NuChek-Prep, Elysian, Minn.), [^{14}C]arachidonic acid and [^{32}P] phosphoric acid (Amersham, Arlington Heights, Ill.). Trifluoperazine and trifluoperazine sulfoxide were kindly donated by Dr. Harry Green, of Smith Kline & French Laboratories (Philadelphia, Pa.).

RESULTS

Effect of trifluoperazine on platelet aggregation. In platelets obtained from more than 40 different donors, trifluoperazine effectively antagonized platelet aggregation produced by a wide variety of aggregating agents, such as collagen, thrombin, trypsin, epinephrine, the bee venom polypeptide melittin, and ionophore A23187. In addition, aggregation induced by exogenous arachidonic acid or PGH_2 was also inhibited by trifluoperazine (Fig. 1). Trifluoperazine sulfoxide was ineffective. For all aggregating agents the degree of inhibition of aggregation was a function of the concentration of trifluoperazine, but the effectiveness of trifluoperazine was also inversely proportional to the stimulus strength (i.e., concentration of aggregating agent). For example, as shown by representative experiments in Fig. 1, 12.5 μM trifluoperazine completely blocked second-phase aggregation produced by 1.6 μM A23187, but it required 50 μM trifluoperazine to produce a comparable inhibition of 3.2 μM A23187. Higher concentrations of trifluoperazine (100 μM) actually decreased the first phase of aggregation. Aggregation induced by epinephrine, collagen, and arachidonic acid could be completely suppressed by trifluoperazine (Fig. 1). Other factors also affected the response to trifluoperazine, such as the ratio of drug to protein present and the temperature. The drug was less effective when the concentration of platelets or plasma was increased. Trifluoperazine was also more effective at 21° and 31° than at 37°. Platelet shape change induced by thrombin was not affected by concentrations of trifluoperazine that inhibited aggregation (Fig. 2A). Trifluoperazine by itself induced some shape change, but this could be avoided by adding the drug gradually at 5- to 10-μM increments over a period of about 5 min. The onset of the antiaggregating action of trifluoperazine was very rapid (Fig. 2B). Unlike agents which increase intracellular cyclic AMP (e.g., prostaglandins E_1 and I_2), trifluoperazine, at concentrations which blocked aggregation, did not cause substantial disaggregation of platelets when it was added after maximal aggregation had occurred.

Effects of trifluoperazine on secretion. Secretion was assessed by measuring the release of calcium and *N*-acetylglucosaminidase. In platelets obtained from eight different donors, trifluoperazine (but not trifluoperazine

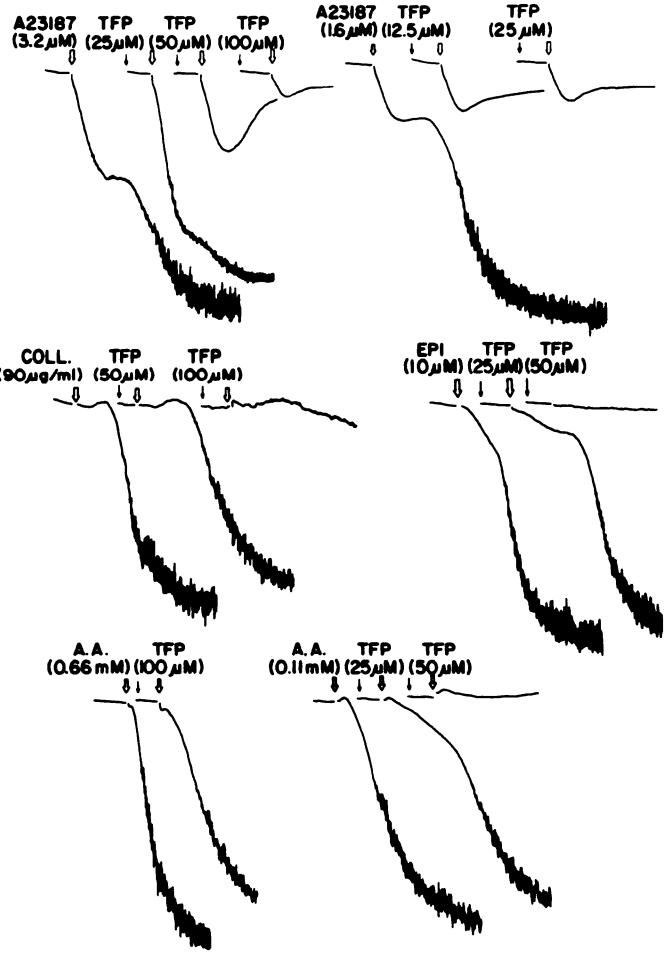

FIG. 1. *Effects of trifluoperazine (TFP) on platelet aggregation due to A23187, collagen (COLL.), epinephrine, and arachidonic acid (A.A.)*

Platelet concentrates were diluted to give a concentration of 1–2 × 10^8 cells/ml at pH 8.0 and 37°. Final drug concentrations in the bath are shown.

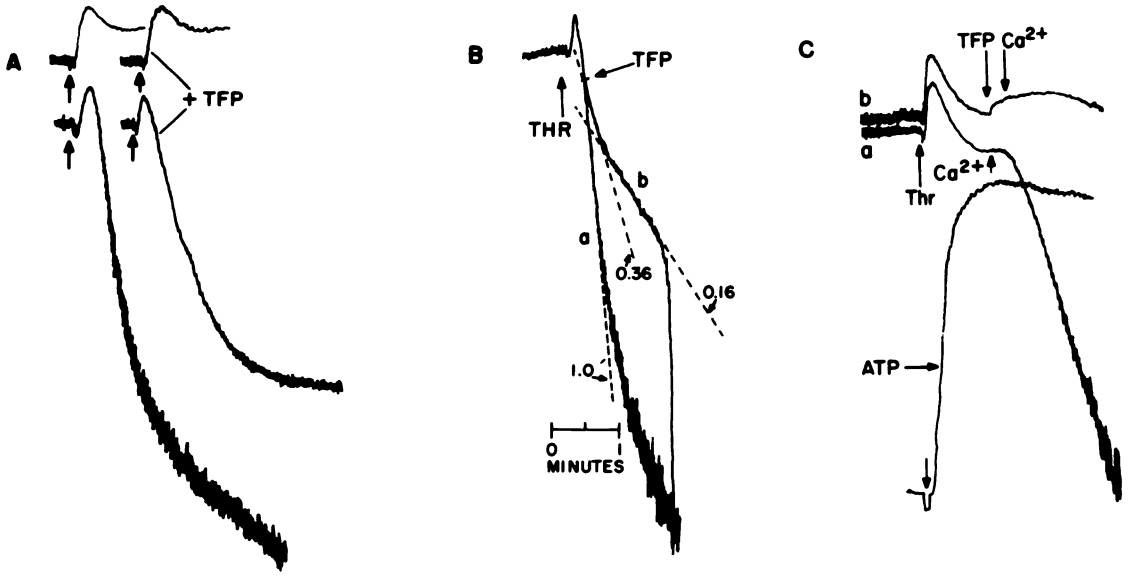

FIG. 2. *Effects of trifluoperazine (TFP) on thrombin (Thr)-induced shape change and aggregation*

A. *Upper records*: shape change (upward deflection) induced by thrombin (0.05 unit/ml) added at *arrows*. The first record is the control response; the second record was obtained in platelets pretreated (2 min) with 50 μM TFP.

B. Aggregation induced by thrombin (0.4 unit/ml): *a*, control; *b*, TFP was added at a concentration of 50 μM in the bath (at *arrow*) after shape change had occurred and aggregation had commenced. *Broken lines* show extrapolated slopes of transmittance changes. *Numbers* refer to the relative slopes with control *a* set at 1.0. The sharp downward deflection at the end of trace *b* is due to clotting.

C. Platelet suspensions in incubation medium containing 2 mM EGTA to prevent aggregation. Shape change occurs after the addition of thrombin (0.4 unit/ml); ATP secretion was measured simultaneously. After 1 min, calcium (2.0 mM final concentration) was added to permit aggregation to occur. In one tube TFP (50 μM final concentration) was added 30 sec prior to calcium. Platelet concentrates (50 μl) were added to 0.45 ml of Tris-buffered salt solution at pH 8.0 to give a final platelet concentration of 1.55×10^8 cells/ml. Temperature was 37°.

sulfoxide) inhibited both the rate and extent of secretion of calcium (measured by atomic absorbance) and *N*-acetylglucosaminidase. The initial rate of secretion was reduced even when the total amount of calcium or enzyme released was not affected (Fig. 3). Trifluoperazine was a considerably more effective inhibitor of secretion (as well as aggregation) at pH 8.0 than at pH 7.4 probably because its uptake into the cell increases as the pH approaches the pK_a of the drug, which is about 8.1. With the use of the Ca^{2+} electrode, which affords the advantage of a continuous record of the secretory process, the initial exponential rate of secretion was always reduced more drastically than the total yield (Fig. 4). As the trifluoperazine concentration was raised, the total amount of calcium secreted was progressively decreased and could even be totally suppressed. Trifluoperazine was able to inhibit secretion induced by any of the stimulating agents that were used, i.e., collagen, thrombin, trypsin, A23187, arachidonic acid, PGH_2, thimerosal, and the bee venom peptide melittin.

Simultaneous measurements of secretion and aggregation: effects of trifluoperazine. When aggregation and secretion of ATP were measured simultaneously in platelets from 20 donors it was observed that trifluoperazine inhibited both processes concomitantly. This effect was noted with all of the aggregating agents studied, although at times one process was affected to a somewhat greater degree than the other. Figure 5 shows representative experiments in which thrombin, collagen, or arachidonic acid was the stimulating agent. It was usual for aggregation induced by thrombin to be somewhat more susceptible to inhibition by trifluoperazine than secretion of ATP. This is in accord with the findings of White and Raynor (7), who measured the effect of trifluoperazine on total serotonin secretion. However, trifluoperazine could inhibit the rate of ATP secretion at concentrations which did not affect the total amount of ATP released (Fig. 5). Arachidonic acid-induced aggregation and ATP release were also inhibited by trifluoperazine (Fig. 5). This effect is especially significant since the metabolism of free arachidonic acid is not affected by trifluoperazine (16).

Since the processes of secretion and aggregation in the platelet tend to enhance each other, we explored the effects of trifluoperazine on each process independently. In one set of experiments, aggregation induced by thrombin in platelets from three different donors was prevented by chelating extracellular calcium with EGTA. Secretion still occurred under these conditions and it was inhibited by trifluoperazine. When secretion was permitted to occur as above and the medium was then recalcified after 1 min to permit aggregation to occur, the addition of trifluoperazine just prior to calcium strongly inhibited the ensuing aggregation (Fig. 2C). It should be noted that trifluoperazine was added at a time when the bulk of the secretion and the release of arachidonic acid from phospholipids had already occurred (see representative experiments in Fig. 2C). Therefore trifluoperazine was able to inhibit platelet aggregation and secretion, each independently of the other.

Effect of trifluoperazine on release of intracellular membrane calcium and secretion. Chlortetracycline is a fluorescent probe for membrane-bound divalent cations and has been used to measure translocation of Ca^{2+} in

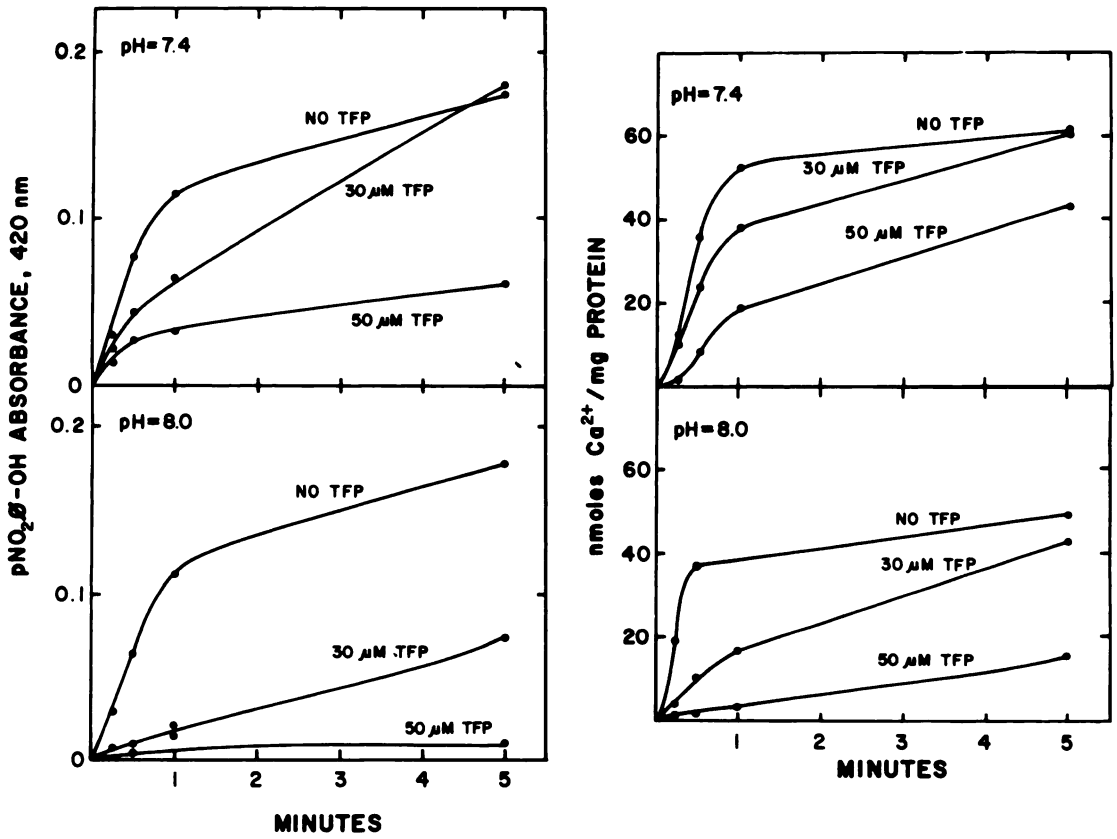

FIG. 3. *Effects of trifluoperazine (TFP) on secretion of N-acetylglucosaminidase and calcium from washed platelets at pH 7.4 and 8.0*
The stimulus in these experiments was 0.2 μM trypsin. Similar results were obtained with thrombin. TFP was added 5 min prior to trypsin. *N*-Acetylglucosaminidase activity was measured by the formation of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. A 1.0 M solution of *p*-nitrophenol at pH 10.5 has an absorbance of 1.62×10^4 at 420 nm. Calcium released into the medium was measured per milligram of platelet protein by atomic absorbance spectroscopy.

intact cells (17, 18), platelets (19), and isolated sarcoplasmic reticulum membranes (20). Using a method developed in our laboratory for the continuous measurement of CTC fluorescence and secretion simultaneously it was shown that a fall in fluorescence commenced almost immediately upon addition of thrombin or trypsin to platelets, and several seconds prior to the onset of secretion as measured with an extracellular Ca^{2+} electrode (12). Furthermore, the CTC fluorescence signal was substantial before secretion had even commenced and was often completed when secretion was only 50% or less of maximum. According to this and other evidence, it was concluded (12) that CTC fluorescence was signalling a very early event in platelet activation—probably the release of intracellular Ca^{2+}—which then triggered the ensuing biochemical reactions leading to aggregation, secretion, and mobilization of arachidonic acid. This method was used to determine whether trifluoperazine interfered with the release of activator Ca^{2+}, which might account for its actions on secretion and aggregation. On the contrary, in platelets from 10 different donors it was found that at concentrations which significantly inhibited secretion, trifluoperazine either had no effect on the CTC signal, or in fact usually increased the initial rate at which intracellular Ca^{2+} was released, with little or no effect on extent of release (Fig. 6). In experiments carried out at pH 8.0 with relatively low concentrations of washed platelets, the more rapid release of intracellular calcium at 10 μM trifluoperazine was sometimes associated with a slightly earlier onset of secretion; however, the initial rate of secretion soon slowed drastically and the total amount of calcium released into the medium was reduced, e.g., by 33% in Fig. 6. At somewhat higher concentrations of trifluoperazine (10–20 μM), the magnitude of the stimulus-induced fall in CTC fluorescence was unchanged or only slightly reduced and the signal of the rate of release of intracellular calcium was either significantly faster or unaffected; but both the rate and extent of secretion were inhibited by about 50–70% (Fig. 6). Thus, under these conditions trifluoperazine appeared to inhibit secretion at a step beyond the initial stage of Ca^{2+} mobilization. At concentrations of trifluoperazine above 30–50 μM the initial rate of intracellular calcium release (CTC fluorescence) was normal, but the magnitude was reduced by about 50%. In this case secretion was affected even more severely, being reduced by at least 75–95% (Fig. 6).

Effect of trifluoperazine on protein phosphorylation. Stimulation of washed platelets, prelabeled with $^{32}PO_4$, by thrombin increased the ^{32}P content of polypeptides from 2- to 6-fold in several regions of the SDS-polyacrylamide gels (16 experiments). The most prominent phosphorylated polypeptides were of the following apparent molecular weights ($\times 10^3$): 20, 47, 71.5, 93.5, 123 and 130 (Figs. 7 and 8). The M_r 20,000 polypeptide migrated identically with myosin LC_{20} purified in our laboratory.

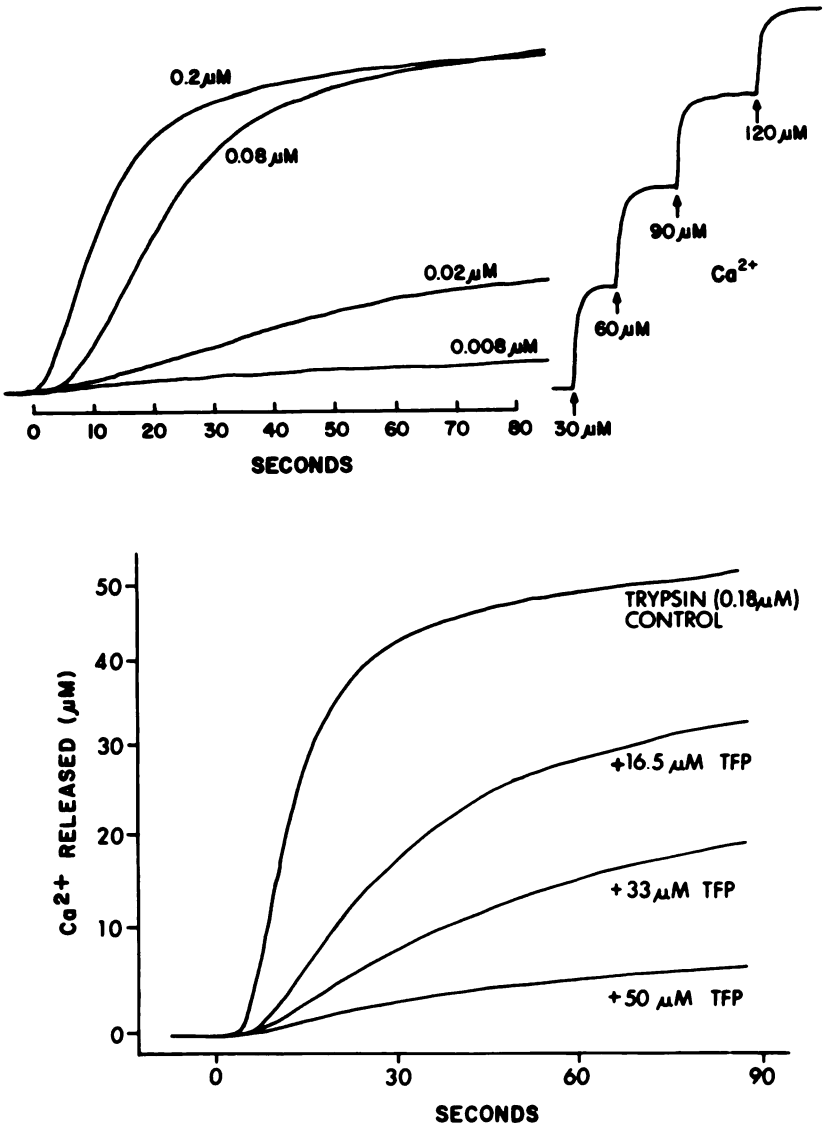

FIG. 4. *Inhibition of calcium secretion from washed platelets by trifluoperazine (TFP)*

Upper record shows calcium electrode response to stimulation of platelets by trypsin at concentrations of 8–200 nM. The calibration response of the electrode to additions of Ca^{2+} standards to the platelet suspension (unstimulated) is also shown. *Lower record*: inhibition of trypsin-induced secretion of calcium. The platelet concentration was 1.0 mg of platelet protein per milliliter of incubation medium, at pH 8.0 (10^9 platelets equal 1.8 ± 0.2 (SE) mg of protein). Temperature was 21°.

Incorporation of ^{32}P into LC_{20} has been shown to be due to net synthesis of the phosphorylated polypeptide rather than increased turnover of an existing pool of phosphorylated myosin (21). The M_r 47,000 polypeptide exhibited the most striking increase in ^{32}P content upon stimulation with thrombin (Figs. 7 and 8). After the addition of thrombin a lag period of about 5 sec was observed prior to the onset of secretion. Very little protein phosphorylation occurred during this lag period. Ninety per cent or more of the ^{32}P incorporation into the M_r 47,000 polypeptide occurred between 5 and 60 sec after addition of thrombin (Fig. 8). The time course for phosphorylation of the M_r 20,000 polypeptide was similar, although it was not extensively studied.

Trifluoperazine (50–100 μM) inhibited the phosphorylation of all polypeptide species (Fig. 7 and 8); however, the effect on the M_r 47,000 polypeptide was often the most pronounced. Inhibition of thrombin-induced ^{32}P incorporation into proteins was usually complete at a trifluoperazine concentration of 100 μM (Fig. 7). The degree of inhibition of protein phosphorylation by trifluoperazine was enhanced as the concentration of the drug relative to the cell protein concentration increased.

DISCUSSION

The underlying assumption in these studies was that trifluoperazine might be employed as a specific antagonist of calmodulin to probe the role that this modulator protein plays in platelet function. This appeared to be a reasonable approach since investigations conducted in other types of cells have established that certain calmodulin-dependent enzymatic reactions can be inhibited by this drug in intact cells (5).

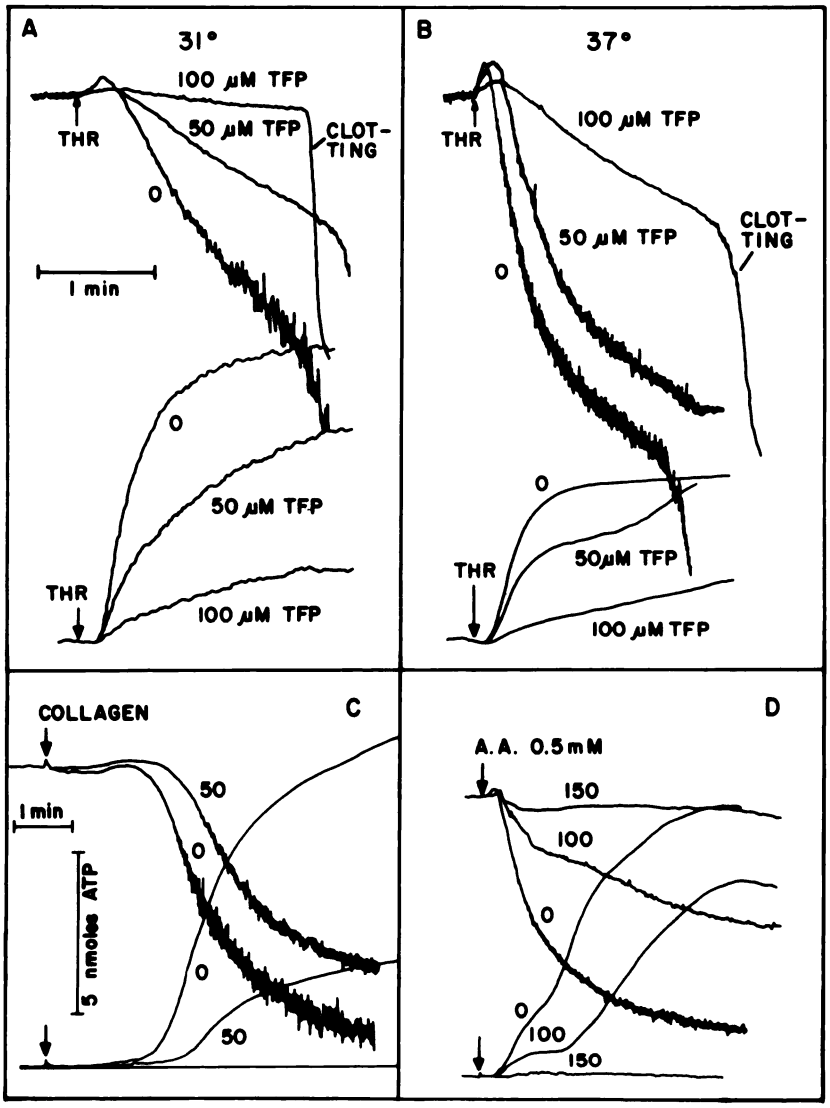

FIG. 5. *Inhibition of ATP secretion and aggregation by trifluoperazine (TFP)*

Platelet concentrates in plasma were diluted into medium at pH 7.4 to a concentration of 2×10^8 cells/ml.

A and B. Stimulation of platelets by thrombin at 31° and 37°. *Upper traces* show aggregation and *lower traces* are simultaneous measurements of ATP secretion in the Lumiaggregometer. The gain of the amplifier for ATP detection was 2 times greater in the experiments at 31° than at 37°. TFP was added 2 min prior to thrombin (0.4 unit/ml).

C and D. Aggregation and ATP secretion induced by collagen and arachidonic acid in diluted platelet concentrates at pH 7.4 and 37°.

Our experiments presented previously (9) and in this paper, and those of Kindness *et al.* (6), Rao *et al.* (8), and White and Raynor (7), demonstrate that trifluoperazine is a powerful inhibitor of platelet aggregation. We have shown that aggregation can be inhibited without interference with shape change and even after normal secretion and arachidonic acid metabolism have been allowed to occur. Furthermore, trifluoperazine, which was previously shown to inhibit the secretion of serotonin (7), can also inhibit the release of Ca^{2+} and ATP, which are constituents of the dense granules, as well as the secretion of a constituent of lysosomes, *N*-acetylglucosaminidase. Secretion occurring when aggregation is prevented by the presence of EGTA can also be inhibited by trifluoperazine. Thus, secretion and aggregation can be inhibited by trifluoperazine totally independently of each other. Trifluoperazine antagonized all of the platelet-stimulating agents that were employed, i.e., thrombin, trypsin, collagen, epinephrine, A23187, thimerosal, mellitin, PGH_2, and arachidonic acid. This suggests that trifluoperazine acts on essential biochemical pathways leading to secretion and aggregation that are common for all stimuli. The two biochemical responses believed to involve calmodulin that we have investigated are the mobilization of arachidonic acid and protein phosphorylation.

The mobilization of arachidonic acid from phospholipid may occur by at least three pathways (22): (a) via phospholipase A_2 cleavage of phospholipids (especially phosphatidylcholine and phosphatidylethanolamine); (b) through hydrolysis of phosphatidylinositol to diglyceride by a phosphatidylinositol-specific phospholipase C, followed by the conversion of that product to free arachidonic acid by diglyceride lipase; or (c) through release of

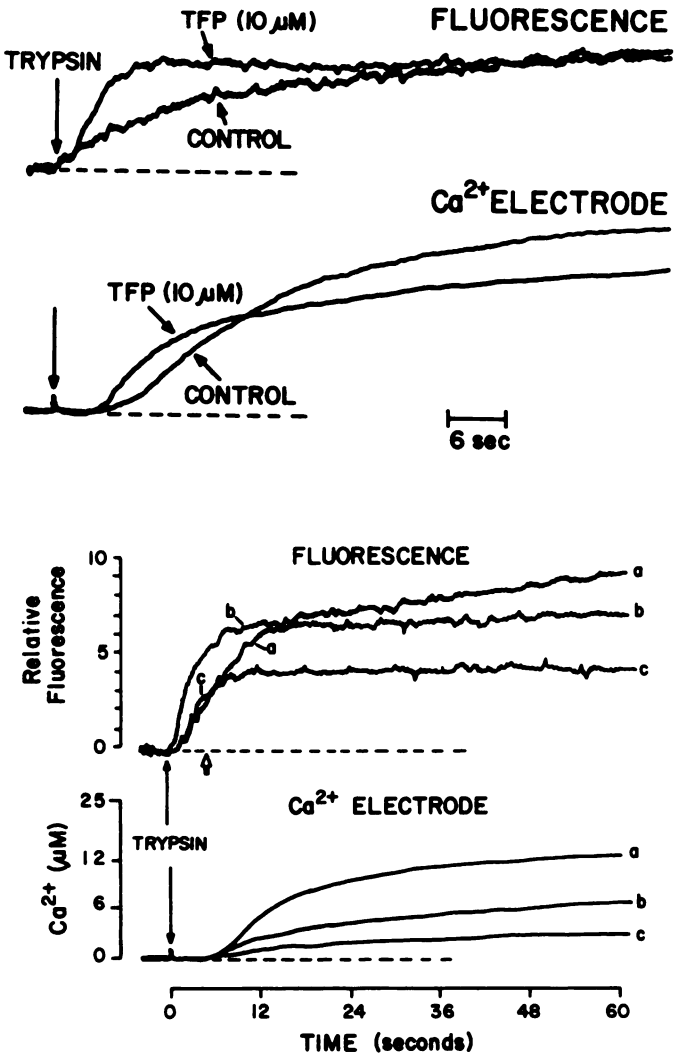

FIG. 6. *Simultaneous measurements of calcium secretion (Ca^{2+} electrode) and release of intracellular calcium (CTC fluorescence)*

Stimulation of platelets causes a fall in fluorescence, but the signal is shown as an upward deflection for ease of comparison with the electrode trace. The trypsin concentration was 0.2 μM; temperature 21°; pH 8.0. *Upper record*: enhanced rate of intracellular Ca^{2+} release in the presence of trifluoperazine (*TFP*) was accompanied by decreased total secretion of calcium. *Lower record*: *a*, control; *b*, +10 μM TFP; *c*, +50 μM TFP. *Open arrow* shows time of onset of secretion. Secretion was inhibited 50% by 10 μM TFP, although the initial rate of intracellular Ca^{2+} release (CTC fluorescence) was increased 3-fold. At 50 μM TFP the early time course of the CTC signal was normal but the magnitude was reduced by about 50%, whereas secretion rate and extent was inhibited by 80%.

arachidonic acid from phosphatidic acid by a phospholipase A_2 active at neutral pH and low Ca^{2+} concentration (23). Phospholipase A_2 activity in isolated platelet membranes has been reported to be stimulated by calmodulin (3), whereas no role for the calcium-dependent regulatory protein has as yet been demonstrated for any of the other enzymes. Our recent findings (16) are consistent with the possible involvement of calmodulin in phospholipase A_2 function in the intact cell in response to thrombin. Trifluoperazine (25–100 μM) produced a dose-dependent inhibition of both thrombin-induced loss of phosphatidylcholine and the formation of arachidonic acid metabolites. At 100 μM trifluoperazine, both the breakdown of phosphatidylcholine and the formation of arachidonic acid metabolites were inhibited 90–95%, but the metabolism of exogenous arachidonic acid to thromboxane B_2 and hydroxy acids was unaffected (16). Therefore prostaglandin endoperoxide synthetase, thromboxane synthetase, and 12-lipoxygenase were unaffected by the drug. The phospholipase C-mediated hydrolysis of phosphatidylinositol and subsequent generation of phosphatidic acid was not prevented by trifluoperazine, and at concentrations of trifluoperazine between 10 and 50 μM the extent of phosphatidylinositol breakdown and the amount phosphatidic acid produced were actually increased (16). These effects of low concentrations of trifluoperazine may be associated with the increased rate of mobilization of membrane Ca^{2+} detected with CTC fluorescence, as shown in this paper. Although trifluoperazine did not affect the metabolism of free arachidonic acid, it did block secretion and aggregation induced by PGH_2 or arachidonic acid. Therefore the responses to arachidonic acid and its metabolites, like those of other platelet-aggregating agents, appears to be expressed through essential calmodulin-dependent reactions as well as other mechanisms, such as calmodulin-*independent* protein kinases that are also susceptible to the drug (see below).

The second biochemical action of trifluoperazine we have observed is an inhibition of protein phosphorylation. One type of protein kinase activity that is affected by stimulation of platelets with thrombin, collagen, and A23187 (21) is the highly specific calmodulin-Ca^{2+}-dependent myosin LC_{20} kinase. The ability of the calmodulin antagonist trifluoperazine to inhibit myosin phosphorylation in intact platelets further substantiates the involvement of calmodulin in this reaction in intact cells. The concentrations of trifluoperazine that inhibit this reaction in platelets are comparable to those which antagonize calmodulin-dependent phosphorylation of myosin LC_{20} in smooth muscle. Another calmodulin antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), has recently been shown to inhibit thrombin-induced phosphorylation of myosin LC_{20} (24). Myosin phosphorylation appears to proceed along a somewhat earlier time course than does secretion (3) and may be necessary but not sufficient for secretion to occur. It should be noted that intracellular Ca^{2+} release measured by CTC fluorescence occurs along a time course that precedes protein phosphorylation as well as secretion.

Another protein whose phosphorylation is greatly increased in stimulated platelets is an M_r 40,000–41,000 polypeptide identified by Lyons and Atherton (15). This protein has a molecular weight of 47,170 as determined by sucrose density gradient centrifugation and a molecular weight of 49,000 as determined by gel filtration, and has been differentiated from actin, tubulin, and the Type II regulatory subunit of cyclic AMP-dependent protein kinase (15). Our results are in accord with those of Haslam *et al.* (25), who reported that the major phosphorylated protein (other than M_r 20,000) in stimulated platelets had an apparent molecular weight of 47,000 on SDS-polyacrylamide gels. The variations in results from one laboratory to another on the relative molecular

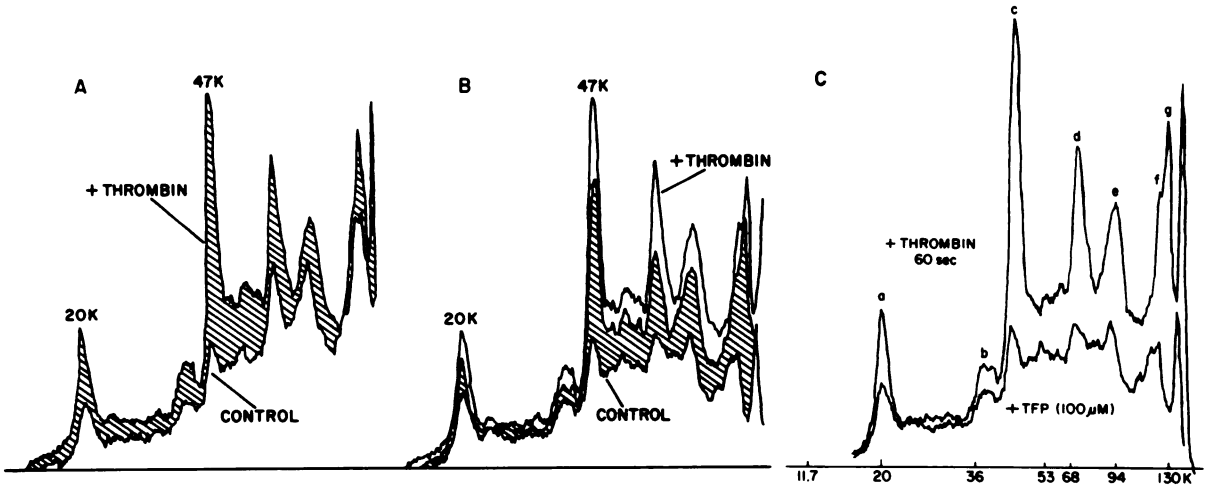

FIG. 7. *Thrombin-induced phosphorylation of platelet proteins*

^{32}P incorporation was determined as described under Materials and Methods. Platelet proteins were separated by SDS-polyacrylamide gel electrophoresis and the gels were exposed to X-ray film. The records shown are the densitometer tracings of the X-ray film.

A. Control platelets and platelets stimulated with thrombin (1.0 unit/ml) (*shaded area*).

B. Control and thrombin-stimulated platelets and thrombin-stimulated plus 50 μM trifluoperazine (*TFP*) (*shaded area*).

C. Inhibition of thrombin-induced incorporation of ^{32}P-phosphate into platelet proteins by TFP. Densitometric trace of X-ray film of electrophoresis gel shows positions of major phosphorylated polypeptides (*a–g*). The apparent molecular weights ($\times 10^3$) are as follows: *a*, 20; *b*, 47; *c*, 71.5; *d*, 93.5; *e*, 123; and *f*, 130. The *upper trace* is the response to thrombin (1 unit/ml). The *lower trace* is that from platelets pretreated with 100 μM TFP 2 min before thrombin. The latter response was almost exactly the same as the control (no thrombin added), which is not shown for the sake of clarity. TFP, Trifluoperazine.

weight of this polypeptide appear to be due to the variable migration of this polypeptide in the different gel systems that have been employed (15). In our experiments, phosphorylation of the M_r 47,000 polypeptide was increased 3- to 6-fold by stimulation with thrombin and occurred mainly during the time span of 5–60 sec after stimulation. The thrombin-induced phosphorylation of M_r 47,000 was strongly inhibited by trifluoperazine. Indeed, that reaction appeared to be somewhat more susceptible to the drug than the phosphorylation of the myosin light-chain.

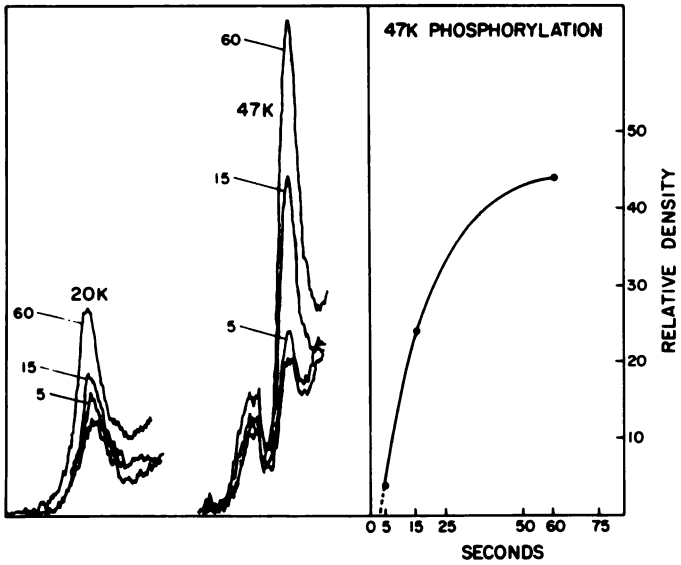

FIG. 8. *Time course of thrombin-induced phosphorylation of M_r 20,000 and 47,000 platelet proteins*

The densitometer traces show the level of radioactivity 5 sec, 15 sec, and 60 sec after the addition of thrombin (1 unit/ml). The phosphorylation of the M_r 47,000 polypeptide (*47K*) is plotted as the increase in radioactivity above the controls as a function of time after addition of thrombin.

The phosphorylation of this protein may be due to a phospholipid- and Ca^{2+}-dependent protein kinase that is widely distributed in animal cells, including platelets, and is known as protein kinase C (26). It catalyzed the phosphorylation of an M_r 40,000 polypeptide in sonicated platelets (27). The sensitivity of this enzyme to Ca^{2+} is considerably enhanced by unsaturated diacylglycerol that can be released from phosphatidylinositol by the enzyme phospholipase C in stimulated platelets. It is therefore conceivable that protein kinase C is activated in the platelet as a result of the combined effects of Ca^{2+} mobilization and the breakdown of phosphatidylinositol initiated by thrombin. Although protein kinase C is not a calmodulin-dependent enzyme it can be inhibited by dibucaine, chlorpromazine (27), and trifluoperazine (26), all of which are also calmodulin-antagonists (4, 28). The drugs antagonize this enzyme apparently by competing with the phospholipid activator. In view of these facts the inhibition by trifluoperazine of stimulus-induced phosphorylation of platelet proteins, other than LC_{20}, cannot be attributed solely to an effect on calmodulin-dependent protein kinases. The absolute specificity of trifluoperazine as a calmodulin antagonist is therefore doubtful, and its use as a probe of the involvement of calmodulin in physiological processes must be assessed with caution. However, it should be noted that there are striking similarities between protein kinase C and some calmodulin-dependent enzymes which may account for their susceptibility to inhibition by the same types of drugs (26–28). Both types of enzymes normally have a common dependence on Ca^{2+}, and several calmodulin-dependent enzymes, as well as protein kinase C, can also

be activated by phospholipids (29, 30). Activation of phosphodiesterase by phospholipid was inhibited by a phenothiazine, which is also a calmodulin antagonist (30). These findings suggest that similar chemical factors are involved in the interaction of these drugs with both phospholipids and calmodulin. Hydrophobic interactions are likely to play an important role.

Since the activities of all calmodulin-dependent enzymes, as well as protein kinase C and phospholipases A_2 and C, require Ca^{2+} it was imperative to determine whether trifluoperazine affected cellular processes by limiting the mobilization of calcium. Our findings indicate that trifluoperazine can inhibit secretion in the platelet without interfering with stimulus-induced release of intracellular Ca^{2+}. In fact, low concentrations of trifluoperazine increased the rate of release of intracellular membrane Ca^{2+} by protease stimulants (i.e., thrombin and trypsin), yet the rate and extent of secretion were markedly reduced. Therefore, as in neutrophils (31), trifluoperazine acts to *uncouple* the secretion response from the initial stages of activation. However, high concentrations of trifluoperazine can reduce the magnitude of intracellular calcium release. The changes in CTC fluorescence appear to represent at least two stages of calcium release (9): an early, rapid release not inhibited by trifluoperazine, and a later stage that can be suppressed by the drug. It is possible that defective Ca^{2+} mobilization is a secondary consequence of the effects of trifluoperazine on lipid metabolism (16) or protein phosphorylation. Alternatively, a primary interference with the release of intracellular Ca^{2+} may account for some of the effects of the drug on protein phosphorylation and lipid metabolism. Cause and effect may be difficult to sort out completely because it is likely that calcium mobilization and some Ca^{2+}-dependent reactions are linked together in a feedback loop. Indeed, calcium mobilization due to Ca^{2+}-activated enzymes provides a plausible mechanism for so-called "Ca^{2+}-induced calcium release."

REFERENCES

1. Klee, C. B., T. B. Crouch, and P. G. Richman. Calmodulin. *Annu. Rev. Biochem.* **49**:489–515 (1980).
2. Harris, H. Regulation of motile activity in platelets, in *Platelets in Biology and Pathology* (J. L. Gordon, ed.), Vol. 2. Elsevier/North-Holland, Amsterdam, 473–500 (1981).
3. Wong, P. Y.-K., and W. Y. Cheung. Calmodulin stimulates human platelet phospholipase A_3. *Biochem. Biophys. Res. Commun.* **90**:473–480 (1979).
4. Weiss, B., and T. L. Wallace. Mechanisms and pharmacological implications of altering calmodulin activity, in *Calcium and Cell Function, Vol. 1: Calmodulin* (W. Y. Cheung, ed.) Academic Press, New York, 330–380 (1980).
5. Kerrick, W. G. L., P. E. Hoar, and P. S. Cassidy. Calcium-activated tension: the role of myosin light chain phosphorylation. *Fed. Proc.* **39**:1558–1563 (1980).
6. Kindness, G., F. B. Williamson, and W. F. Long. Inhibitory effect of trifluoperazine on aggregation in human platelets. *Thromb. Res.* **17**:549–554 (1980).
7. White, G. C., and S. T. Raynor. The effects of trifluoperazine, an inhibitor of calmodulin, on platelet function. *Thromb. Res.* **18**:279–284 (1980).
8. Rao, G. H. R., K. R. Reddy, and J. G. White. Influence of trifluoperazine on platelet aggregation and disaggregation. *Prostaglandins Med.* **5**:221–234 (1980).
9. Feinstein, M. B., and R. W. Walenga. The role of calcium in platelet activation, in *Biochemistry of Acute Allergic Response* (K. F. Austen and E. L. Becker, eds.). Alan R. Liss, New York, 279–293 (1981).
10. Miller, J. L., A. J. Katz, and M. B. Feinstein. Plasmin inhibition of thrombin-induced platelet aggregation. *Thromb. Diath. Haemorrh.* **33**:286–309 (1975).
11. Setkowsky, C. A., B. Lages, H. J. Day, H. J. Weiss, and M. C. Scrutton. Content and thrombin-induced release of acid hydrolyses in gel-filtered platelets from patients with storage pool disease. *Blood* **48**:131–142 (1975).
12. Feinstein, M. B. Release of intracellular membrane-bound calcium precedes the onset of stimulus-induced exocytosis in platelets. *Biochem. Biophys. Res. Commun.* **93**:593–600 (1980).
13. Feinman, R. D., J. Lubowsky, I. Charo, and M. P. Zabinisky. The lumi-aggregometer: a new instrument for simultaneous measurement of secretion and aggregation by platelets. *J. Lab. Clin. Med.* **90**:125–129 (1977).
14. Wallace, W. W., and H. B. Bensusan. Protein phosphorylation in platelets stimulated by immobilized thrombin at 37° and 4°C. *J. Biol. Chem.* **255**:1932–1937 (1980).
15. Lyons, R. M., and R. M. Atherton. Characterization of a platelet protein phosphorylated during the thrombin-induced release reaction. *Biochemistry* **18**:544–552 (1979).
16. Walenga, R. W., E. E. Opas, and M. B. Feinstein. Differential effects of calmodulin antagonists on phospholipases A_2 and C in thrombin-stimulated platelets. *J. Biol. Chem.* **256**:12523–12528 (1981).
17. Chandler, D. E., and J. A. Williams. Intracellular divalent cation release in pancreatic acinar cells during stimulus-secretion coupling. *J. Cell Biol.* **76**:371–385 (1978).
18. Naccache, P., M. Volpi, H. J. Showell, E. L. Becker, and R. I. Sha'afi. Chemotactic factor-induced release of membrane calcium in rabbit neutrophils. *Science (Wash. D. C.)* **203**:461–463 (1979).
19. LeBreton, G. C., R. J. Dinerstein, L. J. Roth, and H. Feinberg. Direct evidence for intracellular divalent cation redistribution associated with platelet shape change. *Biochem. Biophys. Res. Commun.* **71**:362–370 (1976).
20. Caswell, A. H., and B. C. Pressman. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292–298 (1972).
21. Daniel, J. L., H. Holmsen, and R. S. Adelstein. Thrombin-stimulated myosin phosphorylation in intact platelets and its possible involvement in secretion. *Thromb. Haemostasis* **38**:964–969 (1977).
22. Rittenhouse-Simmons, S., and D. Deykin. Release and Metabolism of arachidonate in human platelets, in *Platelets in Biology and Pathology* (J. L. Gordon, ed.), Vol. 2. Elsevier/North-Holland, Amsterdam, 349–372 (1981).
23. Billah, M., and E. G. Lapetina. Degradation of platelet phosphatidic acid by a specific phospholipase A_3. *Fed. Proc.* **40**:337 (1981).
24. Nishikawa, M., T. Tanaka, and H. Hidake. Ca^{2+}-calmodulin-dependent phosphorylation and platelet secretion. *Nature (Lond.)* **287**:863–865 (1980).
25. Haslam, R. J., J. A. Lynham, and J. E. B. Fox. Effects of collagen, ionophore A23187 and prostaglandin E_1 on the phosphorylation of specific proteins in blood platelets. *Biochem. J.* **178**:397–406 (1979).
26. Kuo, J. F., R. G. G. Anderson, B. C. Wise, L. MacKerlova, I. Salomonsson, N. L. Brackett, N. Katoh, M. Shoji, and R. W. Wrenn. Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipid, calmodulin, and trifluoperazine. *Proc. Natl. Acad. Sci. U. S. A.* **77**:7039–7043, (1980).
27. Kawahara, Y., Y. Takai, R. Minakuchi, K. Sano, and Y. Nishizuka. Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. *Biochem. Biophys. Res. Commun.* **97**:309–317 (1980).
28. Volpi, M., R. I. Sha'afi, and M. B. Feinstein. Antagonism of calmodulin by local anesthetics: inhibition of calmodulin-stimulated calcium transport of erythrocyte inside-out membrane vesicles. *Mol. Pharmacol.* **20**:363–370 (1981).
29. Niggli, V., E. S. Adunyah, J. T. Penniston, and E. Carafoli. Purified (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane: reconstitution and effect of calmodulin and phospholipids. *J. Biol. Chem.* **256**:395–401 (1981).
30. Wolff, D. J., and C. O. Brostrom. Calcium-dependent cyclic nucleotide phosphodiesterase from brain: identification of phospholipids as calcium-independent activators. *Arch. Biochem. Biophys.* **173**:720–731 (1976).
31. Naccache, P. H., T. F. P. Molski, E. L. Becker, H. J. Showell, and R. I. Shaafi. Calmodulin inhibitors block neutrophil degranulation at a step distal from the mobilization of calcium. *Biochem. Biophys. Res. Commun.* **97**:62–68 (1980).

Send reprint requests to: Dr. M. B. Feinstein, Department of Pharmacology, University of Connecticut Health Center, Farmington, Conn. 06032.