Serine Esterase Inhibitors Block Stimulus-induced Mobilization of Arachidonic Acid and Phosphatidylinositol-specific Phospholipase C Activity in Platelets*

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Ronald Walenga, Jack Y. Vanderhoeck, and Maurice Feinberg

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

Serine esterase inhibitors (phenylmethanesulfonyl fluoride, 5-dimethylaminophthalene-1-sulfonyl (dansyl) fluoride, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, or p-nitrophenyl anthranilate) blocked the production of malonyldialdehyde by platelets induced with a variety of stimuli (including thrombin, trypsin, collagen, and A23187). These inhibitors did not block malonyldialdehyde production by platelets from exogenous arachidonic acid. Those inhibitors studied in greater detail (phenylmethanesulfonyl fluoride and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate) were shown to inhibit the release of [1-14C]arachidonic acid from phosphatidylinositol and phosphatidylcholine in intact platelets but not the conversion of arachidonic acid to thromboxanes, prostaglandins, or hydroxy fatty acids. These inhibitors also blocked the stimulus-induced production of [32P]phosphatidic acid in intact platelets. Both arachidonic acid release from phosphatidylinositol and phosphatidic acid production have been reported to depend on the production of diglyceride by the action of a phosphatidylinositol-specific phospholipase C. That enzyme in the soluble fraction of disrupted platelets was inhibited at concentrations of serine esterase inhibitors which block arachidonic acid release in intact platelets. These results indicate that serine esterase inhibitors block the stimulus-induced mobilization of arachidonic acid in platelets at least in part by their action on the phosphatidylinositol-specific phospholipase C.

Metabolites of arachidonic acid are important to platelet function. Thromboxane A2, as well as prostaglandin, can induce platelet aggregation and the release of serotonin from storage granules (1). Thromboxane A2 is also a potent vasoconstrictor and the hydroxy acids formed by the enzymatic action of lipoxygenase (i.e. HETE) and thromboxane synthetase (i.e. HHT) may be chemotactic agents (2). Since there is little or no free arachidonic acid in the platelet, regulation of the production of these metabolites is presumed to be exerted on the enzymatic release of arachidonic acid from the platelet phospholipids. Mechanisms for release of arachidonic acid involving phospholipase A2 (3, 4) or the sequential action of a phosphatidylinositol-specific phospholipase C and diglyceride lipases (5-8) have been proposed.

The release of arachidonic acid from platelet phospholipids is stimulated by a variety of platelet aggregating agents, such as collagen, thrombin, and certain other proteases (e.g., trypsin and papain), basic peptides and mellitin, certain thiol reagents, and the divalent cation ionophore A23187. We previously reported that in platelets stimulated by collagen or thrombin, the serine esterase inhibitor PMSF blocked formation of malonyldialdehyde (9). Exoenzyme A2, as well as N,N-dimethylphenylalanine-4-carboxyphenyl-N,N-diphenylcarbamate and p-nitrophenylanthranilate and suggest the phosphatidylinositol-specific phospholipase C as a site for their action.

**MATERIALS AND METHODS**

Platelets were obtained less than 24 h after collection from the Connecticut American Red Cross Blood Center and were concentrated to 20 to 30 mg/ml of platelet protein in their own plasma. Where indicated, platelets were washed as previously described (10) and suspended at a concentration of 20 to 30 mg/ml of platelet protein in their own plasma.

Platelet phospholipids were prelabeled with [1-14C]arachidonic acid essentially as described by Bills et al. (11). Exogenous arachidonic acid metabolism was measured at a total concentration of 50 μM arachidonic acid with 1 to 3 mg/ml of washed platelets in 1 ml of buffer. In all experiments where radioactive products were determined, the reactions were stopped by the addition of 10 ml of chloroform/methanol (1:1, v/v) plus 0.5 ml of 0.5 M citric acid, pH 3. Fractions of the lipid extract were analyzed by thin layer chromatography for arachidonic acid and metabolites and phospholipids (12). Radioactivity in bands from thin layer plates was determined by direct counting in a beta counter (RPI), 12% water, 6% methanol.

Phosphatidic acid formation was measured in platelets which were prelabeled for 60 min with 5 μCi of [32P]phosphatidic acid in 5 ml of plasma. They were then harvested, washed, and resuspended. Platelets were stimulated in pH 7.4 buffer (plus 1 M CaCl2) with collagen (70 μg/ml), thrombin (10 units/ml), or trypsin (4 μM) either without inhibitor or in the presence of either 1 mM 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate or 2 μM PMSF. After 2 min at 37°C, lipids were extracted with chloroform/methanol (2:1, v/v) and analyzed by TLC in the organic phase of ethyl acetate/triethylamine/acetic acid/water (110:50:20:100, v/v), a system in which phosphatidic acid is the only phospholipid which migrates (13).

Platelet supernatant fractions of sonicated cells were prepared for assay of phosphatidylinositol-specific phospholipase C activity essentially as described by Mauro et al. (7). 0.75 mg of supernatant protein was incubated with 200 μM phosphatidylinositol (yeast, Avanti...
at different times and with platelets from different donors. Each
let protein is set at Me2SO.

and diluted 10-fold into platelet suspensions. Controls contained 10

stopped and malonyldialdehyde measured as previously described (9).

was isolated from the lipid extract
stopped by the addition of 2.5 ml of chloroform/methanol (2:1, v/v)
and quantitated by gas-liquid chromatography of fatty acid
methyl esters produced by transesterification with 10% boron tri-
chloride in methanol.

Malonyldialdehyde and platelet oxygen consumption were deter-
mained as previously described (9).

RESULTS AND DISCUSSION

Malonyldialdehyde production was chosen for the initial assess-
ment of the effect of serine esterase inhibitors since this
colorimetrically assayable compound arises from the metab-
olism of prostaglandin endoperoxides, probably largely by the
action of thromboxane synthetase on PGH2 (14). Several
different chemical types of serine esterase inhibitors were used
in these experiments. These inhibitors are compounds with
well defined specificity for the active site serines of certain
estersases and proteases. For example, 2-nitro-4-carboxy-
phenyl-N,N-diphenylcarbamate in common with other p-ni-
 trophenyl carbanates (15), reacts stoichiometrically with chy-
motrypsin to form inactive diphenylcarbamyl-chymotrypsin
(16); PMSF and other sulfonyl fluorides sulfonate the active
site serine of trypsin, chymotrypsin, and thrombin (17, 18);
dansyl fluoride reacts specifically at the active sites of chy-
motrypsin and subtilisin (19); and p-nitrophenylthranilate
has been used as a specific reporter group for the active site
of chymotrypsin (20). The results presented in Table I illus-
rate that each of these inhibitors, as well as others related to
them, is capable of antagonizing the stimulus-induced produc-
tion of malonyldialdehyde by platelets, but had little or no effect
on the production of malonyldialdehyde from 25 to 300 M
exogenous arachidonic acid. The inhibition of stimulus-in-
duced malonyldialdehyde formation by the compounds listed
in Table I was concentration-dependent. Nearly complete
inhibition of stimulus-induced malonyldialdehyde production
could usually be attained when the inhibitor concentrations
were 2 to 3 times higher than shown in Table I. The inhibitors
were also effective in blocking malonyldialdehyde production
induced by polylysine, mellitin, and y-globulin-coated latex
beads (data not shown).

We have been able to rule out interactions between the
serine esterase inhibitors and the platelet-stimulating agents
as a trivial cause for the observed effects. For example, PMSF
sulfonates the active site serine of thrombin (21), and PMS-
thrombin is inactive as a stimulant of platelet aggregation
(22). We were able to obviate this problem by employing
supramaximal concentrations of thrombin (10 to 20 units/ml)
that, within the time required to carry out an experiment
(i.e. 5 min), only partial inactivation of thrombin occurred (9)
and sufficient thrombin activity remained intact to produce
maximal activation of platelet arachidonic acid metabolism
when added to normal platelets. Papain is partially inhibited
by PMSF, but can be completely protected by thiols such as
cysteine (23). Cysteine-activated (1 M) papain treated with
PMSF, ABSF, or 2-nitro-4-carboxyphenyl-N,N-diphenylcar-
bamate retained its activity against the chromogenic substrate
o-N-benzoyl-DL-arginine-p-nitroanilide. When trypsin was
used as the platelet stimulant, neither 2-nitro-4-carboxy-
phenyl-N,N-diphenylcarbamate nor dansyl fluoride inacti-

TABLE I
Effects of serine esterase inhibitors on malonyldialdehyde
production from exogenous and endogenous arachidonic acid

Platelets concentrated in plasma to 20 to 30 mg of platelet protein/ 
mL were diluted 5 to 10-fold in Tris-buffered saline at pH 8.0, 37°C.
Inhibitors were added for 2 min followed either by arachidonic acid
or one of the platelet stimulants. After 5 min, the reactions
were stopped and malonyldialdehyde measured as previously described (9).

| Inhibitor | Arachidonate 50 μM | A23187 9 μM | V. Edefsulemaleate 5 mM | Trypsin 4 μM | Collagen 70 μg/mL | Papain 10 μM |
|-----------|---------------------|------------|------------------------|-------------|----------------|-------------|
| Control   | 100                 | 100        | 100                    | 100         | 100            | 100         |
| PMSF 2 mM | 92                  | 67         | 53                     | 16          | 25             | 9           |
| ABSF 4 mM | 100                 | 0          | 0                      | 1            | 0              | 0           |
| NCDC 5 mM | 100                 | 0          | 0                      | 1            | 0              | 0           |
| Dansylfluoride 1 mM | 97 | 87 | 9 | 13 | 0 | 0 |
| NPA 2 mM | 96                  | 32         | 43                     | 16          | 0              | 0           |

All inhibitors were dissolved in dry dimethyl sulfoxide (Me2SO)
and diluted 100-fold into platelet suspensions. Controls contained 1% Me2SO.

The control production of malonyldialdehyde in nmol/mg of plate-
let protein is set at 100 to normalize all results which were conducted
at different times with platelets from different donors. Each
result is expressed relative to its own control.

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NCDC: 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate.

TABLE II
Differential effects of serine esterase inhibitors on trypsin versus
platelets

| Pretreatment of | Malonyldialdehyde production |
|----------------|-----------------------------|
| Trypsin        | Platelets                   |
| None           | None                        | 100                      |
| NCDC 5 mM      | None                        | 100                      |
| None           | DANSyl fluoride 1 mM         | 4                        |
| None           | DANSyl fluoride 0.01 mM      | 15                       |

"Control platelets (1 to 2 mg of protein/ml) were incubated with 4 
M control trypsin (no inhibitor present) for 5 min at 37°C and
malonyldialdehyde production was measured. This value was set at 100.

"Trypsin at 400 μM was incubated with indicated concentrations of 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) or dansyl
fluoride for 5 min at 37°C. Aliquots of the pretreated trypsin were
then diluted 100-fold into suspensions of normal platelets resulting in
inhibitor concentrations of 30 μM NCDC and 10 μM dansyl fluoride
experienced by the platelets. Note that little inhibition of malonyldi-
aldehyde production occurred under these conditions.

Platelets were pretreated with the indicated concentration of inhibitor for 2 min at 37°C. Untreated trypsin (4 μM) was then added
for 5 min and malonyldialdehyde was measured. Note that under
these conditions (i.e. preincubation of the inhibitors with the platelets
rather than with trypsin), more than 90% inhibition of malonyldi-
aldehyde production was attained.
particular step in its metabolism was the site of action. This was substantiated by experiments in platelets whose phospholipids were prelabeled with radioactive arachidonic acid. Upon subsequent stimulation by collagen (24), by proteases such as thrombin or papain (Fig. 1), or by A23187 (Fig. 2), 20 to 50% of the [14C]arachidonic acid in phosphatidylcholine (the major pool of labeled arachidonic acid) and a similar proportion of that in phosphatidylinositol was released. [14C]Arachidonic acid in phosphatidylethanolamine and phosphatidylserine was essentially unchanged. PMSF or 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate inhibited the decrease of radioactive arachidonic acid from platelet phospholipid and the subsequent formation of radioactive arachidonic acid metabolites induced by papain (Fig. 1), and thrombin (20 units/ml) (data not shown). The increases in the content of phosphatidylcholine and phosphatidylinositol induced by collagen, papain (Fig. 1), A23187 (Fig. 2), or thrombin (20 units/ml) (data not shown). The increases in the

**Fig. 1.** Inhibition by PMSF of the release of [14C]arachidonic acid from platelet phospholipid and the subsequent formation of [14C]arachidonic acid metabolites induced by papain. Platelet phospholipids were labeled with [14C]arachidonic acid. The washed cells were then stimulated with papain (10^-5 m) and cysteine (1 mm) for 5 min. The cells were extracted and radioactivity in phospholipids and products was analyzed as described under "Materials and Methods." PMSF-treated cells were incubated in the presence of 2 mM of that inhibitor for 2 min prior to the addition of papain and 1 mm cysteine. Values are averages of duplicate samples from at least two different batches of platelets. PC, phosphatidylcholine; PI, phosphatidylinositol.

**Fig. 2.** Inhibition by 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) of the release of [14C]arachidonic acid from platelet phospholipid and the subsequent formation of [14C]arachidonic acid metabolites induced by A23187. Platelet suspensions prelabeled with [14C]arachidonic acid were stimulated with 10 μM A23187 in the presence or absence of 1 mm NCDC. The [14C] content of phosphatidylcholine (PC) and phosphatidylinositol (PI) as well as the formation of [14C] metabolites and malondialdehyde (MDA) were all measured on the same platelet batches.

radioactive metabolites of arachidonic acid (TXB₂, HHT, and HETE) due to stimulation were also inhibited by PMSF (Fig. 1) and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) were added at the indicated concentration immediately before the addition of papain and 1 mm cysteine. Values are averages of duplicate samples from at least two different batches of platelets. PC, phosphatidylcholine; PI, phosphatidylinositol.

**Fig. 3.** Inhibition of phosphatidic acid (PA) formation. Platelets were prelabeled with [32P]phosphoric acid as described under "Materials and Methods," harvested, washed, and resuspended to 4 mg of platelet protein/ml. Platelets were stimulated with 70 μg of collagen without inhibitor (a), with 1 mm 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (b), or 2 mm PMSF (c). The phosphate-labeled material with B₅ - 0.1 co-migrated with authentic phosphatidic acid. No such labeled material was seen in extracts from non-stimulated platelets.

**Fig. 4.** Inhibition of phospholipase C. Hydrolysis of phosphatidylinositol by platelet supernatant fractions was measured as described under "Materials and Methods." The inhibitors PMSF and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) were added at the indicated concentration immediately before the addition of phosphatidylinositol.
by thrombin, trypsin or collagen with a large increase in $[^{32}P]$phosphatidic acid (13). Phosphatidic acid appears to be formed by the phosphorylation of the diglyceride produced by the action of phospholipase C on phosphatidylinositol. PMSF and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (each at 1 mM) strongly inhibited the formation of $[^{32}P]$phosphatidic acid in intact, stimulated platelets (Fig. 3); inhibition was greater than 90% with collagen and 50 to 75% with thrombin (10 units/ml) or trypsin (4 μM). In view of this finding, we investigated the effects of PMSF and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate on phospholipase C activity in soluble fractions obtained from disrupted platelets. PMSF and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate inhibited phospholipase C hydrolysis of phosphatidylinositol over the same concentration range found to be effective in intact platelets (Fig. 4).

**DISCUSSION**

The mechanisms by which various stimuli induce the release of arachidonic acid from platelet phospholipids have not been unequivocally demonstrated. It has been presumed for some time that arachidonic acid is released by the action of a phospholipase A₂ on platelet phospholipids (3, 4). An alternative pathway for arachidonic acid mobilization has recently been proposed (6, 8). The earliest event in lipid metabolism in stimulated platelets appears to be the hydrolysis of phosphatidylinositol to diglyceride by the action of a phosphatidylinositol-specific phospholipase C (5). Bell et al. (6) have demonstrated the presence of diglyceride lipase in platelets which acts on the arachidonate-rich diglyceride produced from phosphatidylinositol, resulting in net formation of free arachidonic acid. While this mechanism does not account for the hydrolysis of phosphatidylinositol, it may represent a critical early phase in arachidonic acid release. Another event in stimulated platelets which appears to precede arachidonic acid release is the production of phosphatidic acid (13). No specific inhibitors are known for either diglyceride lipase or the diglyceride kinase which catalyzes production of phosphatidic acid and the relative importance of these two possible fates of diglyceride in the platelet has not been conclusively established.

In this report, we have demonstrated that compounds which inhibit the common first enzyme of these pathways (i.e. phosphatidylinositol-specific phospholipase C) block not only phosphatidic acid formation and release of arachidonic acid from phosphatidylinositol but also the release of arachidonic acid from phosphatidylcholine. While it remains to be demonstrated that the serine esterase inhibitors do not directly block other enzymes involved in these metabolic pathways (i.e. phospholipase A₂, diglyceride lipase, or diglyceride kinase), our findings are consistent with the proposed central role of phospholipase C in the stimulus-induced mobilization of platelet arachidonic acid. Since arachidonate release from phosphatidylcholine is also blocked by these inhibitors, it is possible that some product of phosphatidylinositol metabolism (e.g. diglyceride, arachidonic acid or its metabolites, or phosphatidic acid) is an activator of the putative phospholipase A₂. Furthermore, since the serine esterase inhibitors block arachidonic acid mobilization by a wide range of stimulating agents, the target(s) of the inhibitors must be common to all stimulatory pathways. Mobilization of intracellular Ca²⁺ is likely to be important in the release of arachidonic acid since this cation is an activator of both phospholipase A₂ (3) and phosphatase C (5, 8). However, since serine esterase inhibitors block arachidonic acid release brought about by A23187, which presumably releases intracellular Ca²⁺ by a direct action as an ionophore, it appears that an enzymatic step beyond Ca²⁺ mobilization is blocked.

We conclude, therefore, that 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate and PMSF can block formation of free arachidonic acid in platelets, in part at least, by inhibiting the phosphatidylinositol-specific phospholipase C. This is the first class of enzyme inhibitors which has been shown to affect this important enzyme. Further studies with the serine esterase inhibitors should be useful for investigating the enzymatic properties of the phosphatidylinositol-specific phospholipase C and for establishing in more detail the initial enzymatic pathways for arachidonic formation in platelets.

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