Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 502-508

Inhibition of Binding of the Platelet-Activating Factor AGEPC to Platelets by the AGEPC Analog rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate (CV-3988)[1]

Frank H. Valone

Department of Medicine (111 H), Veterans' Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121

Received November 30, 1984

SUMMARY: CV-3988, rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate, is a specific inhibitor of the platelet-activating activity of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine (AGEPC or $PAF_{acether}$). Concentrations of CV-3988 between 10^{-8} and 10^{-7} M inhibited AGEPC-induced aggregation of washed human platelets in a dose-related manner ($IC_{50} = 2.9 \pm 1.1 \times 10^{-8}$ M CV-3988) whereas concentrations of CV-3988 as high as 4×10^{-6} M did not diminish platelet aggregation by thrombin or adenosine diphosphate. The binding of [^{3}H] AGEPC to platelets was inhibited by CV-3988 in a concentration-dependent manner ($IC_{50} = 6.7 \pm 1.8 \times 10^{-8}$ M). Compared to AGEPC, CV-3988 has a 1000-fold lower affinity for the AGEPC receptor. CV-3988 did not stimulate platelet metabolism of AGEPC as assessed by thin-layer chromatographic analysis of [^{3}H] AGEPC extracted from platelet suspensions after four hours of incubation. Thus these studies indicate that CV-3988 inhibits platelet activation by AGEPC by inhibiting binding of AGEPC to its specific platelet receptor.

The phospholipid 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine (AGEPC)[2] is a potent platelet-activating factor which is the product of many model IgE-mediated and immune complex-mediated immunological reactions (1). Recent studies have established that AGEPC activates platelets by interaction with specific, high affinity platelet membrane receptors (2). Further analysis of the interaction of AGEPC with its receptor has been hampered, however, by the lack of specific AGEPC antagonists. Thus the demonstration by Terashita et al. that rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate (CV-3988) is a specific antagonist of AGEPC-induced platelet activation is of great interest (3). The current studies of the

[1] This work was supported in part by grant AI 19784 from the National Institutes of Health, and grants MSC 06 TROUP and MSC 07 BLAIR from the University of California.

[2] Abbrevations used are: ADP, adenosine diphosphate; AGEPC, 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine; PBS, phosphate-buffered saline, pH 7.2; PBS-HSA, PBS containing 0.1% (w/v) human serum albumin; PBS-HSA-Ca^{++}, PBS-HSA containing 1.8 mM $CaCl_2$.

0006-291X/85 $1.50

mechanism of action of CV-3988 demonstrate that this agent blocks AGEPC binding to its specific platelet receptor.

METHODS AND MATERIALS

[^{3}H] AGEPC (1-O-alkyl-1',2'-3H-,45 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and unlabeled AGEPC was prepared as described (2). Both products were purified by high performance liquid chromatography on an amino-spherisorb (Phase-Sep, Hauppage, KY) column which was developed with acetonitrile : water (85:15, v/v) at a flow rate of 1 ml/min. CV-3988 was the generous gift of Dr. Allen Wissner (Lederle Laboratories, Pearl River, NY) and was prepared as described (3).

Platelet Isolation and Quantitation of Platelet Aggregation and Free Intracellular Calcium. Platelets were obtained from healthy adult volunteers who had not taken any medications for 14 days before venipuncture. The platelets were isolated and washed by centrifugation onto cushions of autologous erythrocytes as described (2). For analysis of platelet aggregation, platelets were suspended at a concentration of 2×10^8/ml of phosphate-buffered saline (pH 7.2) containing 0.1% (w/v) human serum albumin (Sigma Chemical Co., St. Louis, MO) and 1.8 mM $CaCl_2$ (PBS-HSA-Ca^{++}). Four-tenths milliliter aliquots of platelets were prewarmed at 37°C in siliconized cuvettes and were then stirred for 1 min in an aggregometer. CV-3988 in 1 µl of ethanol or 1 µl of ethanol alone was then added to the suspension and 1 min later the platelets were stimulated by the addition of AGEPC in 1 µl of methanol, or by the addition of thrombin or ADP (Sigma Chemical Co., St. Louis, MO) in 1 µl of PBS-HSA. Aggregation was quantitated as the maximal percentage increase in light transmission with 100% increase in transmission defined as the maximal increase induced by 0.5 U/ml of thrombin. Changes in free intracellular calcium were quantitated using the fluorescent dye Quin 2 (4,5).

Quantitation of AGEPC Binding. To quantitate AGEPC binding, platelets were suspended at a concentration of 1×10^8/ml in PBS-HSA-Ca^{++}. One milliliter of the suspension was then added to microfuge tubes which contained [^{3}H] AGEPC (2,200 cpm) without or with excess unlabeled AGEPC, CV-3988, or the appropriate buffer. The final concentration of organic solvents in each sample was maintained at 0.5% (v/v). After incubation for 10 min at room temperature, the platelets were sedimented by centrifugation in a microfuge for 1 min (Beckman Instruments, Palo Alto, CA). The supernatants were transferred to scintillation vials for quantitation of unbound [^{3}H] AGEPC; the pellets were resuspended in 0.5 ml of 1% sodium dodecylsulfate and then transferred to scintillation vials for quantitation of bound [^{3}H] AGEPC. Binding was quantitated as the percentage of total recovered radioactivity which was in the platelet pellet.

RESULTS

Initial studies demonstrated that CV-3988 inhibits platelet aggregation by AGEPC in a dose-related manner (Table 1). In four experiments, concentrations of CV-3988 of 20 nM or greater inhibited aggregation significantly ($p < 0.05$, paired T-test) and the IC_{50} was $2.9 \pm 1.1 \times 10^{-8}$ M CV-3988 (mean $\pm$ SD). This concentration represents a 50- to 400-fold molar excess of CV-3988 over the concentration of AGEPC used to induce aggregation in each experiment (243 ± 98, mean $\pm$ SD). The specificity of inhibition is shown by the failure of 100-fold higher concentrations of CV-3988 to diminish platelet

Table 1

Specific Inhibition of AGEPC-induced Platelet Aggregation by CV-3988

Concentration of CV-3988	Stimulus	Percent Aggregation
0*	AGEPC	33†
10 nM	AGEPC	36
20 nM	AGEPC	27
40 nM	AGEPC	19
100 nM	AGEPC	15
200 nM	AGEPC	5
400 nM	AGEPC	0
0	Thrombin	54
4 μM	Thrombin	52
0	ADP	68
4 μM	ADP	63

* Platelets were incubated with CV-3988 or buffer for 1 min in the aggregometer and were then stimulated with 1.0 nM AGEPC, 0.01 U/ml thrombin, or 10 μM ADP.

† Mean maximal percentage aggregation for two determinations. The data are representative of four studies performed.

aggregation by 0.1 U/ml of thrombin or by 10 μM ADP. Four micromolar CV-3988 induced 9 $\pm$ 3% platelet aggregation (mean $\pm$ SD, n = 4) whereas lower concentrations which completely blocked platelet activation by AGEPC had no direct platelet aggregating activity. CV-3988 also blocked the capacity of AGEPC to increase free intracellular calcium with significant inhibition ($p < 0.05$, paired T-test) achieved by 2.0 μM and greater CV-3988 (Figure 1). Fifty percent inhibition was induced by a 180-fold molar excess of CV-3988 in two experiments, which is approximately the same molar ratio as was observed for inhibition of aggregation.

In order to examine the mechanisms by which CV-3988 inhibits platelet activation by AGEPC, the capacity of CV-3988 and unlabeled AGEPC to compete with [^{3}H] AGEPC for binding to platelets was compared (Figure 2). As expected, concentrations of unlabeled AGEPC between 10^{-10} and 10^{-9} M inhibited binding of [^{3}H] AGEPC in a dose-related manner. CV-3988 also inhibited [^{3}H] AGEPC binding in

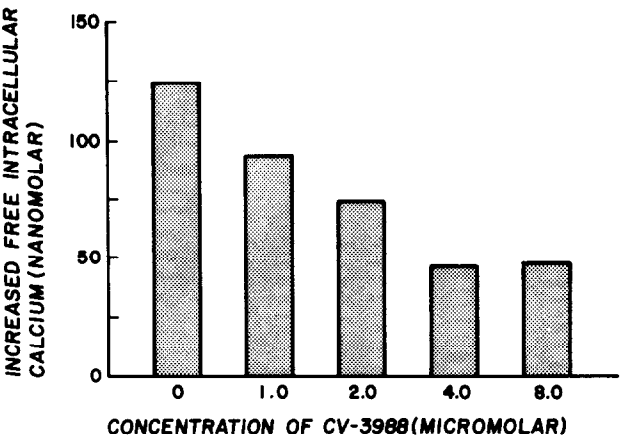

Figure 1. Inhibition of AGEPC-mediated increased intracellular calcium by CV-3988. Platelets were pretreated with CV-3988 or buffer for 1 min and were then stimulated with 16 nM AGEPC. The data are the mean of two determinations of the increase in free intracellular calcium induced by AGEPC. Free intracellular calcium was 109 nM in unstimulated platelets.

a dose-related manner, but at 100- to 1000-fold higher concentrations. Non-specific binding accounts for 30% of total [^{3}H] AGEPC binding (Ref. 3, Figure 2). Thus, 50% inhibition of specific [^{3}H] AGEPC binding was achieved by 7.1 $\pm$ 1.9 x 10^{-11} M unlabeled AGEPC and 6.7 $\pm$ 1.8 x 10^{-8} M CV-3988 (mean $\pm$ SD, n = 5). Having demonstrated that CV-3988 inhibits AGEPC binding to platelets, the capacity of CV-3988 and unlabeled AGEPC to dissociate previously bound [^{3}H] AGEPC was

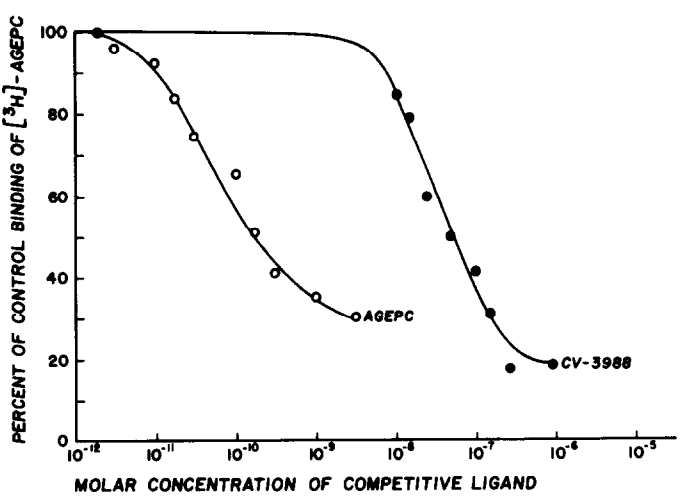

Figure 2. Inhibition of [^{3}H] AGEPC binding to human platelets by unlabeled AGEPC and CV-3988. [^{3}H] AGEPC binding in the absence of inhibitors was 34.2%. Each point is the mean of replicate determinations which varied less than 10% from the mean. The data are representative of five similar experiments.

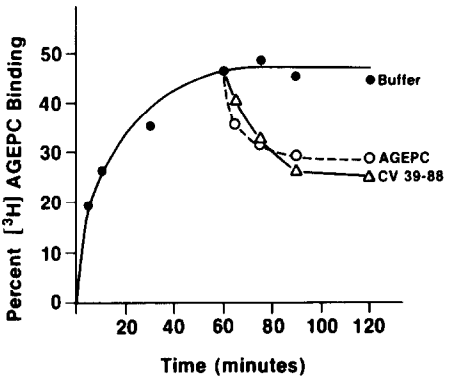

Figure 3. Reversal of [^{3}H] AGEPC binding by unlabeled AGEPC and CV-3988. Sixty-four x 10^6 platelets in 32 ml PBS-HSA-Ca^{++} were mixed with 250,000 cpm of [^{3}H] AGEPC. Two 0.5 ml aliquots were removed for quantitation of [^{3}H] AGEPC binding at the indicated times. After 60 min of incubation, the remaining platelets were transferred in 0.5 ml aliquots to microfuge tubes containing buffer (●), 4 μM CV-3988 (△), or 2.5 nM unlabeled AGEPC (○). At the indicated times, [^{3}H] AGEPC binding was assessed in replicate samples. The supernates and pellets from additional replicates were saved at -70°C for analysis of AGEPC metabolism.

assessed (Figure 3). [^{3}H] AGEPC was allowed to bind to platelets for 60 min. Replicate 0.5 ml aliquots of platelets were then mixed with buffer, excess unlabeled AGEPC or excess CV-3988 and binding was quantitated after further incubation for 1 to 240 min. In three experiments, [^{3}H] AGEPC binding was reversed 64 $\pm$ 15% by excess AGEPC and 59 $\pm$ 7% by excess CV-3988 ($p < 0.01$, for both blockers, paired T-test). Half-maximal dissociation of bound [^{3}H] AGEPC occurred 6.0 $\pm$ 3.9 min and 10.5 $\pm$ 4.7 min after addition of AGEPC and CV-3988 respectively (p = NS, paired T-test).

The modulation of AGEPC metabolism by CV-3988 was assessed by thin-layer chromatographic analysis of the [^{3}H] AGEPC in replicate supernates and platelet pellets obtained after incubation for 10 or 240 min in the studies described in Figure 3. The samples were first extracted twice with two volumes of chloroform : methanol (2:1, v/v). More than 90% of the radioactivity recovered was in the lower chloroform phase. This material was then subjected to thin-layer chromatography on silica gel H plates which were developed with chloroform : methanol : water, (65:35:6, v/v/v). The gel was then scraped from the plates in 0.5 cm strips and each strip assessed for radioactivity. A single peak of radioactivity was identified for supernates and platelet pellets obtained at both time points from buffer-, CV-3988-, and AGEPC-treated platelets. This peak coincided with the [^{3}H] AGEPC marker, indicating that no substantial metabolism of AGEPC occurred under any of the incubation conditions.

DISCUSSION

These studies demonstrate that the AGEPC analog rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate (CV-3988) specifically inhibits human platelet aggregation (Table 1) and increased free intracellular calcium induced by AGEPC (Figure 1). CV-3988 has no direct platelet-activating activity, except at concentrations > 1 μM, which are 10- to 50-fold greater than the concentrations which fully block AGEPC-induced platelet aggregation, and 5000-fold greater than the concentration of AGEPC which induces comparable aggregation. Thus, CV-3988 is principally an AGEPC antagonist. Platelet aggregation by AGEPC was inhibited 50% by $2.9 \pm 1.1 \times 10^{-8}$ M CV-3988 which is substantially lower than the IC_{50} observed by Kinoshita et al. (3). The use of platelet-rich plasma to assess aggregation in the latter study accounts for the difference. A 30-fold higher concentration of AGEPC was required to induce aggregation of platelets in plasma. Thus, in both studies, platelet aggregation was inhibited 50% at a molar ratio of CV-3988 and AGEPC of 200.

Analysis of the mechanisms by which CV-3988 inhibits the actions of AGEPC revealed that CV-3988 blocks binding of AGEPC to its receptor (Figure 2). [^{3}H] AGEPC binding was inhibited 50% by $6.7 \pm 1.8 \times 10^{-8}$ M CV-3988 and by $7.1 \pm 1.9 \times 10^{-11}$ M unlabeled AGEPC, suggesting that CV-3988 has a 1000-fold lower affinity for the AGEPC receptor than does AGEPC. Similarly, both AGEPC and a 1,000-fold higher concentration of CV-3988 displaced approximately 60% of previously bound [^{3}H] AGEPC at comparable rates (Figure 3). That inhibition of binding is the principal mechanism by which CV-3988 blocks the actions of AGEPC is suggested by the close correlation between the concentration of CV-3988 which yields 50% inhibition of aggregation (2.9×10^{-8} M) and binding (6.7×10^{-8} M). The observation that only native AGEPC is recovered from platelets and supernates after a 4 h incubation of [^{3}H] AGEPC with platelets without or with excess unlabeled AGEPC or CV-3988 indicates that CV-3988 does not stimulate AGEPC metabolism by platelets and is further evidence that CV-3988 inhibits platelet activation by blocking AGEPC binding. The availability of a specific AGEPC inhibitor will now permit better analysis of the contribution of AGEPC to immunological reactions in vivo and will lead to the

development of additional probes for exploring structure-function relationships for cellular activation by AGEPC.

REFERENCES

1. Pinckard, R.N., McManus, L.M., and Hanahan, D.J. (1982) Advances in Inflammation Research, vol. 4, pp 147-180, Raven Press, New York.
2. Valone, F.H., Coles, E., Reinhold, V.R., and Goetzl, E.J. (1982) J. Immunol. 129, 1637-1641.
3. Terashita, Z., Tsushima, S., Yoshioka, Y., Nomura, H., Inada, Y., and Nishikawa, K. (1983) Life Sci. 32, 1975-1982.
4. Hallam, T.J., Sanchez, A., and Rink, T.T. (1984) Biochem. J. 218, 819-826.
5. Valone, F.H., and Johnson, B. J. Immunol., in press.