Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 68-74

RHC 80267 DOES NOT INHIBIT THE DIGLYCERIDE LIPASE PATHWAY IN INTACT PLATELETS

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Received August 29, 1983

RHC 80267 inhibits diglyceride lipase activity in microsomes from canine platelets (1). Chau and Tai (2) reported that RHC 80267 prevents the transient accumulation of monoglyceride in thrombin-stimulated human platelets, while leaving arachidonate release unimpaired. In contrast, we find that while the drug inhibits both diglyceride lipase (I_{50}=15 μM) and monoglyceride lipase (I_{50}=11 μM) activities in platelet microsomes, it is ineffective when added to intact platelets. The transient intermediates in the diglyceride lipase pathway, 1,2-diglyceride and 2-monoglyceride, both accumulated after thrombin stimulation of intact platelets treated with RHC 80267, and arachidonate release was not inhibitied. We conclude that RHC 80267 cannot be used to evaluate the diglyceride lipase pathway in intact platelets.

The production of eicosanoid metabolites in platelets and other cells is triggered by agonist-induced hydrolysis of arachidonate from cellular phospholipids. When platelets are stimulated with thrombin, there is a rapid fall in phosphatidylinositol levels with the transient appearance of 1,2-diglyceride (3) and 2-monoglyceride (4), suggesting that these substances are intermediates in arachidonate release. The phospholipase C-diglyceride lipase pathway provides a mechanism for the release of arachidonate from phosphatidylinositol (5). Other mechanisms have been formulated to describe arachidonate release from phosphatidylcholine and phosphatidylinositol (6). Estimation of the amount of arachidonate released via the different potential pathways in whole cells has been hampered by the lack of specific inhibitors (7). Sutherland and Amin (1) recently have described an inhibitor, RHC 80267, of diglyceride lipase activity in microsomes from

0006-291X/83 $1.50

canine platelets. Chau and Tai (2) used this drug in an attempt to elucidate the relative contribution of the diglyceride lipase pathway to the release of arachidonate by human platelets. They reported that RHC 80267, added to intact platelets prior to thrombin-stimulation, prevented the formation of the transient 2-monoglyceride, indicating that the drug inhibited diglyceride lipase in intact cells. In the same experiments the drug did not inhibit arachidonate release, implying that the diglyceride lipase pathway is not required for arachidonate release. In contrast to these results, we find that RHC 80267 does not prevent the appearance of either transient intermediate in the diglyceride lipase pathway, suggesting that the drug is inactive when added to intact platelets. Furthermore, in enzymatic assays with microsomes from human platelets, the drug inhibits monoglyceride lipase activity as well as it does diglyceride lipase.

Materials and Methods

Unlabeled fatty acids and neutral lipids were obtained from NuChek (Elysian, MN) and [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (120 mCi/mmole) was purchased from New England Nuclear. Butylated hydroxytoluene was from Shell Chemical (New York). Bovine thrombin was a gift from Dr. Craig Jackson (Washington University). Liquid scintillation spectrometry was performed in a Beckman LS7000 instrument using Scintiverse I (Fisher). Samples of RHC 80267 used were from Dr. C. Sutherland (Revlon Health Care) and Dr. R.R. Gorman (Upjohn). Apiezon oil was from J.B. Biddle Co.

Diglyceride and Monoglyceride Lipase Assays

Radiolabeled substrates and platelet microsomes were prepared as described (4,8). The assays for monoglyceride and diglyceride lipase activities were performed as described (4,8). Briefly, the assay measures the release of radiolabeled fatty acid from the neutral lipid substrate. The fatty acid is extracted into the aqueous upper phase, while neutral lipids are in the organic lower phase. RHC 80267 was added in dimethyl sulfoxide and allowed to incubate with the microsomal preparation for 5 minutes at room temperature before the assays were started by the addition of substrate. Control assays were incubated with an equal volume of dimethyl sulfoxide, which did not affect either activity. In some experiments the lower phase of the extraction mixture was analyzed by one dimensional thin layer chromatography (4). Appropriate areas were scraped into vials and radioactivity determined by liquid scintillation spectrometry.

Experiments with Intact Platelets

Washed human platelets were isolated as previously described (9). They were labeled by adding 8 μCi of [^{3}H]arachidonate in 160 μl of 50 mM sodium bicarbonate (containing 10 mg/ml fatty acid free bovine serum albumin) to 16 ml of buffer containing 26.2 mM sodium and 6.8 mM potassium phosphate pH 6.5, 118 mM NaCl, 5,6 mM glucose and $8x10^{9}$ platelets. After incubation for 30 minutes at 37°C, the platelets were collected by centrifugation for 10 minutes at 2000xg, washed once in the same buffer containing 1 mg/ml fatty acid free albumin and resuspended in 16 ml of buffer without albumin. RHC 80267 was added to the platelets in dimethyl sulfoxide 15 minutes (37°)

before thrombin stimulation. Control platelets were treated with an equal amount of dimethyl sulfoxide (0.1%), which had no effect on the processes studied.

Measurement of Released Arachidonate Metabolites

[^{3}H]arachidonate-labeled platelets ($1x10^8$ in 0.2 ml) were incubated with 10 U thrombin/ml for the times indicated; the platelets were sedimented at 11,500Xg through 300 µl of a 1:15 mix of apiezon oil/n-butyl phthalate (specific gravity 1.046). The supernatant fluid was collected and a portion was counted to determine total radiolabeled metabolites released. The remainder was acidified to pH 5 with 1 N formic acid and extracted twice with 1 ml of ethyl acetate containing 50 µg/ml butylated hydroxytoluene and 0.1 µmole unlabeled arachidonic acid. The extract was concentrated and chromatographed on activated Silica Gel 60 plates (E. Merck) in Solvent System I (10).

Measurement of Monoglycerides and Diglycerides

[^{3}H]arachidonate labeled platelets ($5x10^8$) were incubated with 10 U thrombin in 1 ml. Reactions were stopped by the addition of 5 ml $CHCl_3$: CH_3OH:HCl (100:100:0.6). The phases were separated by adding 1.5 ml of 1 N HCl containing 5 mM EGTA, 0.1 µmole of unlabeled monoolein and diolein, and 50 µg/ml butylated hydroxytoluene. The organic phase was dried, treated with silicic acid and analyzed by two-dimensional chromatography as previously described (4).

Results

Effect of RHC 80267 on Lipase Activity of Platelet Microsomes

We find that RHC 80267 inhibits both diglyceride (I_{50} ~15 µM) and monoglyceride lipase (I_{50} ~11 µM) activities when the drug is added to platelet microsomes, as shown in Figure 1. The hydrolysis of 1,2-diglyceride is an

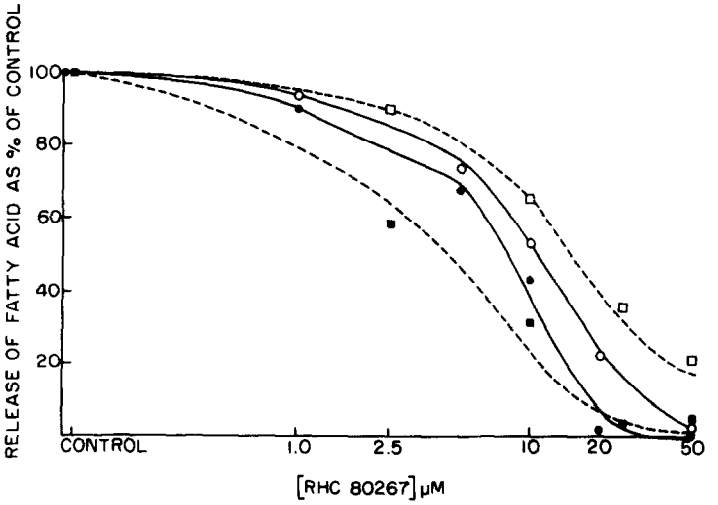

Fig.1. RHC 80267 Inhibits Both Monoglyceride and Diglyceride Lipase Activities in Platelet Microsomes. In separate assays the release of radiolabeled fatty acid from either [^{14}C]2-oleoyl, sn-glycerol (200 µM, ●—●; 400 µM, ○—○) or 1-stearoyl, [^{3}H]2-arachidonoyl, sn-glycerol (200 µM, ■—■; 400 µM, □—□) was measured in the presence of 0-50 µM inhibitor. For monoglyceride lipase 100% activity=7.4 nmol fatty acid released (at 400 µM substrate); for diglyceride lipase 100%=0.6 nmol (at 500 µM). The results are the average of two experiments.

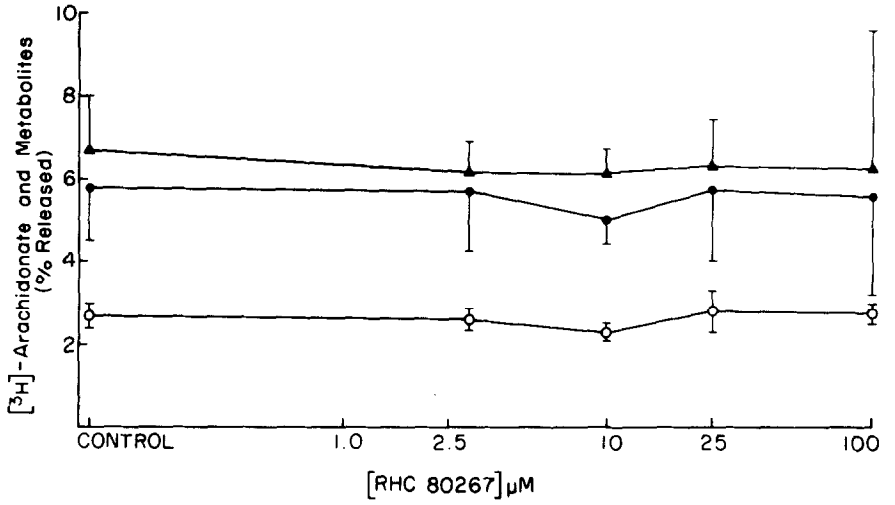

Fig. 2. RHC 80267 Does Not Inhibit Release of Arachidonate Metabolites in Thrombin-Stimulated Platelets. Platelets, labeled with [^{3}H]arachidonate, were resuspended in buffer containing 1 mg/ml fat-free serum albumin, and treated 20 minutes with RHC 80267 in dimethyl sulfoxide before stimulation with thrombin at 23°C for 20 seconds (○), 1 minute (●), or 5 minutes (▲). (See methods) 100%=80,000 cpm. The results are the average of three experiments.

ordered reaction with the fatty acid at sn-1 released first (4). Therefore inhibition of release of the [^{3}H]arachidonate from diglyceride might have resulted from inhibition of only the second step, i.e., monoglyceride lipase. To exclude this possibility we showed that no ^{3}H-monoglyceride accumulated.

Effect of RHC 80267 on Arachidonate Metabolism of Intact Platelets

RHC 80267 has no effect on the amount of arachidonate metabolites released by thrombin-stimulated platelets, as shown in Figure 2. Total release measured after 20 seconds, 1 minute, and 5 minutes, was not inhibited in the presence of 0-100 μM RHC 80267. Release increased from 20 seconds to 1 minute, where it reached 6% of total arachidonate. We have monitored release under a variety of conditions (data not shown); at times ranging from 10 seconds to 5 minutes, using thrombin doses ranging from 0.01 U/ml to 30 U/ml, after preincubation with RHC 80267 ranging from 1 to 20 minutes, in the presence or absence of 1 mg/ml fat free BSA (see figure legend), and with four separate batches of RHC 80267. No significant inhibition of release was obtained under any condition. RHC 80267 does not alter the dis-

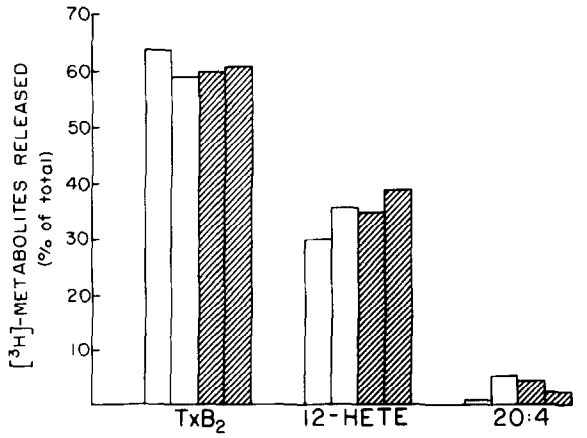

Fig. 3. RHC 80267 Does Not Affect the Distribution of Released Arachidonate Metabolites. Platelets, labeled with [^{3}H]arachidonate, were incubated for 15 minutes at 37°C with dimethyl sulfoxide (□) or 10 μM RHC 80267 (▨) before stimulation with thrombin at 23°C for 1 or 3 minutes. (See methods) 100%= 10,000 cpm.

tribution of arachidonate metabolites released from platelets, as shown in Figure 3. Also, RHC 80267 did not inhibit the production of [^{3}H]thromboxane B_2 from added [^{3}H]arachidonate. These experiments indicate that RHC 80267 does not inhibit thromboxane synthetase or cyclooxygenase.

We measured monoglyceride and diglyceride levels in RHC 80267-treated platelets following stimulation with thrombin, as illustrated in Figure 4.

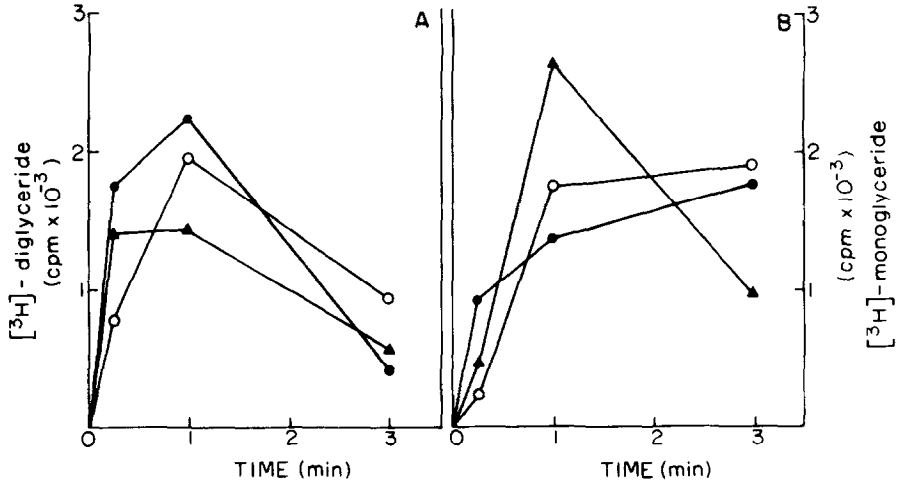

Fig. 4. Effect of RHC 80267 on Monoglyceride and Diglyceride Levels in Thrombin-Stimulated Platelets. Platelets, labeled with [^{3}H]arachidonate, were incubated for 15 minutes with dimethyl sulfoxide (▲), 10 μM RHC 80267 (○) or 50 μM RHC 80267 (●) before stimulation with thrombin at 23°C for the times shown. (See methods) The results are the average of two experiments.

The diglyceride levels in platelets (Figure 4A) treated with 10 μM or 50 μM RHC 80267 are nearly identical to those in control platelets, which exhibit a rise of diglyceride that peaks at 15 seconds and falls to one-fourth of the peak value by 3 minutes.

Monoglyceride was measured in platelets treated with 0, 10 or 50 μM RHC 80267 and stimulated with thrombin (Figure 4B). In controls the level of monoglyceride peaked at 1 minute and fell to one-third of the peak value by 3 minutes. In drug-treated platelets, the level of monoglyceride also rose but did not fall by 3 minutes.

Discussion

Chau and Tai (2) reported that monoglyceride does not accumulate in RHC 80267-treated thrombin-stimulated platelets. Because arachidonate release was not inhibited in these experiments, they concluded that the diglyceride lipase pathway is not essential for arachidonate release. They also found that free arachidonate accumulated upon stimulation. Only 30% of the released products at 2 minutes (8 U/ml thrombin) were thromboxane B_2 and 12-HETE, the remainder being free arachidonate. This product distribution contrasts with our results and previous reports (10,11), which show little or no unmetabolized arachidonate under conditions used in these experiments. It is possible that the platelets used by Chau and Tai (2) were damaged and not only did not produce monoglyceride, but also had decreased metabolism of arachidonate.

We have demonstrated accumulation of labeled monoglyceride in RHC 80267 treated, thrombin-stimulated platelets (Figure 4B). After 3 minutes the amount of labeled monoglyceride present in drug-treated platelets was twice control levels, implying mild inhibition of monoglyceride lipase. However, in the presence of 2-fold higher monoglyceride levels, lipase activity may be normal in intact cells. Thrombin stimulated the transient formation of diglyceride in RHC 80267-treated platelets, suggesting that diglyceride lipase is not inhibited. Chau and Tai (2) also found diglyceride accumulation similar in control and drug-treated platelets. In contrast, Rittenhouse-

Simmons (12) showed that indomethacin, which at high level doses inhibits diglyceride lipase, causes diglyceride to accumulate to levels 5 times those of control platelets 5 minutes after thrombin stimulation.

We have shown that RHC 80267 has little effect on the arachidonate metabolism of intact human platelets, although it inhibits diglyceride and monoglyceride lipase activities in a microsomal preparation. This suggests either the drug does not enter platelets, or it is degraded rapidly, making it ineffective. We conclude that RHC 80267 cannot be used to assess the contribution of the diglyceride lipase pathway to arachidonate release during thrombin stimulation of platelets.

Acknowledgments

We thank Dr. C. Sutherland (Revlon Health Care) and Dr. R. R. Gorman (Upjohn) for the samples of RHC 80267 in these studies. We also thank Ellis J. Neufeld, David B. Wilson and Michael Laposata for helpful discussions concerning this work. This research supported by grants HLBI 14147 (Specialized Center in Thrombosis) and HL 16634 from the National Institutes of Health, and a grant from the Nora Eccles Treadwell Foundation.

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