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EFFECTS OF (1,6-DI (*O*-CARBAMOYL)CYCLOHEXANONE OXIME)HEXANE (RHC 80267) ON PROSTAGLANDIN BIOSYNTHESIS AND ACCUMULATION OF DIACYLGLYCEROL AND ARACHIDONIC ACID IN RABBIT IRIS

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Abstract—The effects of RHC 80267, (1-6-di(*O*-carbamoyl)cyclohexanone oxime)hexane, a diacylglycerol (DG) lipase inhibitor, on the DG lipase pathway and on arachidonic acid (AA) metabolism were investigated in the iris muscle. Incubation of the iris for 30 min at 37° resulted in a loss of AA from phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine of 40, 25, and 32% respectively. It was found that the drug inhibited the activity of DG lipase in the iris microsomal fraction and it increased the accumulation of DG, AA and other glycerolipids in iris muscle prelabeled with [^{14}C]AA, presumably by inhibiting this enzyme. Under the same experimental conditions, the drug increased the accumulation of DG and AA in the tissue in a dose- and time-dependent manner, and it inhibited the synthesis of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ by iris and iris microsomes in a dose-dependent manner. The data presented indicate that RHC 80267 has nonspecific effects on glycerolipid and AA metabolism in this tissue. We conclude that, while the drug does inhibit DG lipase in the intact iris, the present findings that it increased the accumulation of glycerolipids and AA and that it inhibited the biosynthesis of PGs in this tissue throw some doubt on its use in studies on the mechanism of AA release from membrane phosphoinositides for PG synthesis.

In mammalian tissues, arachidonic acid (AA†), the precursor of prostaglandins (PGs), is found mainly esterified to phospholipids and triacylglycerol (TG). It is esterified almost exclusively at the second position of phospholipids [1], from which it could be released by the action of phospholipases [2, 3]. The metabolism of this polyunsaturated fatty acid to PGs has been studied extensively by a number of investigators, including in the iris [4, 5]. Phosphoinositides are enriched in AA, and in some tissues it has been proposed that when degradation of phosphatidylinositol (PI) by a specific phospholipase C to DG occurs, AA is then released from the latter by the DG/monoacylglycerol pathway [6–8]. Sutherland and Amin [9] have described an inhibitor, RHC 80267, (1,6-di-*O*-carbamoyl)cyclohexane oxime) hexane, of DG lipase activity in microsomes from canine platelets, and more recently this drug was used by Chau and Tai [10] and Bross *et al.* [11] in an attempt to evaluate the DG pathway in platelets.

In previous communications from this laboratory, we have investigated the agonist-stimulated phosphodiesteratic breakdown of phosphoinositides (for review see Ref. 12) and the agonist-stimulated release of AA in the iris muscle ([5], and for review see Ref. 13). To throw more light on the mechanism(s) involved in AA release for PG synthesis, we have investigated the effect of RHC 80267 on the DG pathway and on AA metabolism in the iris. We have found that this drug inhibits PG synthesis and it increases the accumulation of DG and AA in this tissue.

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† Abbreviations: AA, arachidonic acid; PG, prostaglandin; DG, diacylglycerol; TG, triacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

MATERIALS AND METHODS

Materials. [1-^{14}C]AA, sp. act. 56.5 mCi/mmole, was purchased from the Amersham Corp., Arlington Heights, IL; and phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl, [arachidonyl-1-^{14}C-], sp. act. 54.5 mCi/mmole, was purchased from the New England Nuclear Corp., Boston, MA. Phospholipase D-phosphatidylcholine phosphohydrolase (EC 3.1.4.4), sp. act. 20 units/mg lyophilisate, was purchased from Pfaltz & Bauer Inc., Stamford, CT. [^{14}C]PA was prepared from [^{14}C]phosphatidylcholine, through the action of phospholipase D, according to Taki and Kanfer [14]. RHC 80267 was a gift from Dr. Charles Sutherland (Revlon Health Care). PGE_2 ^{3}H-RIA kit was obtained from Seragen Inc., Boston, MA. Phospholipids and neutral lipids were from sources described previously [5]. All other chemicals were of reagent grade.

Preparation and incubation of iris muscle. Irises (iris-ciliary body) from cows and albino rabbits were employed in the present study. The iris was removed from the eye and placed in Krebs–Ringer bicarbonate buffer (pH 7.4) that contained 10 mM D-glucose.

In general, two irises from pairs were prelabeled with [^{14}C]AA by incubation in 2 ml of Krebs–Ringer that contained 0.15 μCi of the isotope/ml for 1 hr at 37°. The prelabeled irises were washed three times with nonradioactive medium and then incubated (of the pair one was used as control) in the absence and presence of RHC 80267 as indicated. RHC 80267 was dissolved in 10 μl of absolute alcohol. An equivalent amount of alcohol was routinely added to the controls. The medium was analyzed for PGs and the tissue was analyzed for glycerolipids and AA as previously described [5].

Preparation and incubation of microsomes. Microsomes were prepared from the iris as previously described [15]. Microsomes, equivalent to 0.25 mg protein, were incubated in 1 ml of 0.1 M phosphate buffer, pH 7.8, that contained 1 μM AA, at 37° for 15 min. The reactions were ended by acidification with 10% formic acid to pH 3.5 and addition of ethylacetate.

Extraction and determination of PGs. At the end of incubation the medium was analyzed for PGs. It was acidified with formic acid to pH 3.5 and extracted three times with 3 ml of ethylacetate. The solvent was evaporated under nitrogen. The residue was dissolved in chloroform–methanol (2:1), and PG was determined.

Isolation and determination of ^{14}C-labeled PGs. When [^{14}C]AA was employed as a precursor for PG biosynthesis, an aliquot of the chloroform–methanol extract was spotted on Whatman precoated silica gel LK6DF and developed in a solvent system [5, 16] of diethyl ether–methanol–acetic acid (90:1:2, by vol.). After visualization of the PG standards by exposure to iodine vapour, the radioactive PG spots were located with autoradiography and their radioactive contents were measured by counting in a Beckman Liquid Scintillation Counter.

Determination of PGE_2 by radioimmunoassay. PGE_2 in the chloroform–methanol extract was quantitated using the RIA method of Jaffe *et al.* [17]. The amount of PGE_2 in each sample was determined by interpolation from the standard curve as described previously [18].

Extraction and isolation of glycerolipids. Glycerolipids were extracted twice from the tissue with chloroform–methanol–HCl (400:200:1.5, by vol.), and phospholipids and neutral lipids were separated by TLC. An aliquot of the lipid extract, equivalent to 80% of the total, was used for phospholipid analysis, and the remainder was used for neutral lipid analysis. Phospholipids were separated by means of two-dimensional thin-layer chromatography (TLC) with silica gel H, and their radioactive contents were determined as described previously [19]. Neutral lipids and AA were separated by means of one-dimensional TLC with Whatman precoated silica gel LK6DF plates in a solvent system [5, 20] of hexane–diethyl ether–acetic acid (80:20:1, by vol.). The neutral lipid spots were localized with autoradiography, and their radioactive contents were determined.

Gas–liquid chromatography (GLC). The composition of fatty acids liberated by saponification was determined by GLC of their methylesters as previously described [21].

Protein determination. Protein was estimated by the method of Lowry *et al.* [22] using bovine serum albumin as standard.

Analysis of data. Data are reported as ^{14}C-radioactivity (dpm) incorporated into glycerolipids or converted into PGs/iris, or as ng PGE_2/g tissue, or ng PGE_2/mg protein.

RESULTS

Effect of incubation on AA content of iris phospholipids. In the iris most of the AA is found in PI, PC and PE [21]. Incubation of this tissue for 30 min at 37° resulted in a loss of AA from PI, PC and PE of 40, 25 and 32% respectively (Table 1). These data suggest that in the iris any of these phospholipids could serve as a source of AA for PG synthesis.

Effect of RHC 80267 on glycerolipids of iris prelabeled with [^{14}C]AA. Addition of a 25 μM concentration of the drug to iris muscle prelabeled with [^{14}C]AA provoked an increase in the labeling of glycerolipids which ranged from 17 to 37% of their respective controls (Table 2). Inhibition of DG lipase by the drug could cause accumulation of [^{14}C]DG in the tissue, and this could account for the increase observed in the glycerolipid labeling. Inhibition of AA conversion into PGs by the drug could also increase [^{14}C]AA in the tissue and, consequently, bring about an increase in the labeling of glycerolipids. This interpretation is supported by the

Table 1. Effect of incubation on AA content of bovine iris phospholipids*

Phospholipid	Content of AA (μg/iris) Frozen in liquid N_2	Incubated for 30 min at 37°	% Loss of AA
PI	6.9 ± 0.87 (7)	4.2 ± 0.30 (3)	40
PC	12.4 ± 1.50 (7)	9.3 ± 1.84 (3)	25
PE	11.5 ± 2.10 (7)	7.8 ± 1.86 (3)	32

* One iris from each pair was frozen in liquid N_2 and the other was incubated for 30 min at 37°. Phospholipids were extracted and separated by TLC, and their AA content was determined by GLC as described in the text. Values are means ± S.E. with the number of determinations given in parentheses.

Table 2. Effect of RHC 80267 on the distribution of radioactivity in glycerolipids of rabbit iris muscle prelabeled with [^{14}C]AA*

Glycerolipid	^{14}C-Radioactivity (dpm/iris) Control	+RHC 80267 (25 μM)	% of Control
PA	205	265	129
PI	1426	1811	127
PC	5362	6997	131
PE	979	1226	117
DG	3841	5224	136
TG	6547	8971	137

* Rabbit irises, in pairs, were preincubated in 2 ml of Krebs–Ringer buffer (pH 7.4) that contained 10 mM D-glucose and 0.15 μCi [^{14}C]AA per ml for 1 hr at 37°. The prelabeled irises were washed three times with non-radioactive Krebs–Ringer and then incubated (one of the pair was used as control and the other as experimental) in the absence and presence of RHC 80267 (25 μM) for 30 min. The tissue was analyzed for glycerolipids as described in the text. The results are the average of two experiments.

findings in the present study that RHC 80267 increased the accumulation of [^{14}C]AA in the iris (see below).

Effect of RHC 80267 on DG lipase activity of iris microsomes. This drug is a potent inhibitor of the DG lipase activity of platelet microsomes [9–11]. As can be seen from Table 3, at 25 μM it inhibited the DG lipase activity of iris microsomes by 37% and this was accompanied by a corresponding decrease in the release of labeled AA from [^{14}C]DG. In the assay for DG lipase, the substrate was generated from [^{14}C]PA through the action of microsomal PA-phosphohydrolase [23]. RHC 80267 had no effect on the activity of PA phosphohydrolase (data not shown). In general, the drug increased DG and AA accumulation in the tissue and inhibited PG release in a dose-dependent manner (Fig. 1). Thus, at a 50 μM concentration of the drug, the accumulation of both DG and AA increased by about 70% (Fig. 1A), and the synthesis of PGE_2 and $PGF_{2\alpha}$ decreased by 50 and 35% respectively (Fig. 1B). These data could suggest the following: (a) although RHC 80267

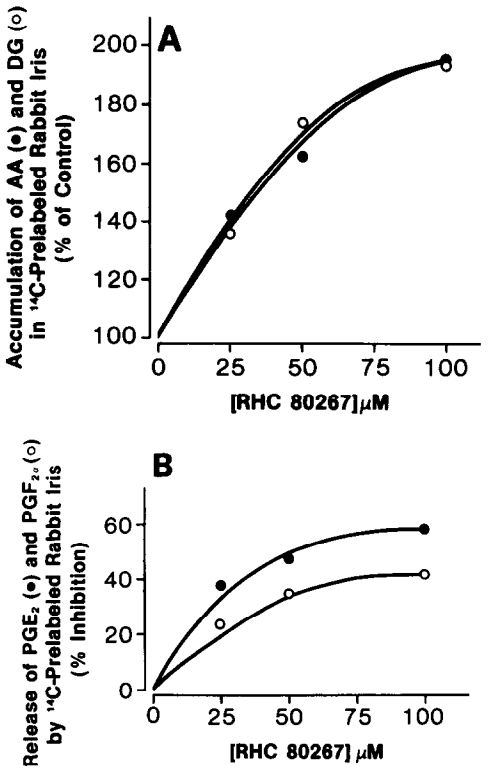

Fig. 1. Effect of RHC 80267 concentration on DG and AA accumulation (A) and PG release (B) in rabbit iris prelabeled with [^{14}C]AA. Conditions of incubation were the same as described under Table 2 except that the irises were incubated in the absence and presence of different concentrations of RHC 80267 as indicated. The tissue was analyzed for DG and AA and the medium was analyzed for PGs as described in the text. The control values (dpm/iris) for AA, PGE_2 and $PGF_{2\alpha}$ were 5284 ± 315, 2214 ± 169 and 1080 ± 89 respectively. The results are the average of two separate experiments.

Table 3. Effect of RHC 80267 on metabolism of [^{14}C]PA and [^{14}C]DG by bovine iris microsomes*

Additions	^{14}C-Radioactivity (cpm) Origin	DG	AA
None	24,671 ± 178	2,588 ± 102	1,516 ± 12
RHC 80267 (25 μM)	25,751 ± 231	3,423 ± 92	887 ± 21

* The assay method for this system is similar to that employed previously for the assay of PA phosphohydrolase [23]. The reaction mixture contained in 0.5 ml: 52 mM Tris–maleate buffer (pH 7.4), 0.4 mM PA (that contained 2.9×10^4 cpm of [^{14}C]PA) added as a sonicated suspension, 200 μg of microsomal proteins (suspended in 0.1 ml of 0.25 M sucrose), and RHC 80267 (25 μM) added as indicated. Incubations were carried out at 37° for 30 min. The products of the reaction were extracted with chloroform–methanol (2:1, v/v), and the formation of [^{14}C]DG and [^{14}C]AA was determined by one-dimensional TLC as described in the text. The data presented are averages of six determinations, and expressed as means ± S.E.

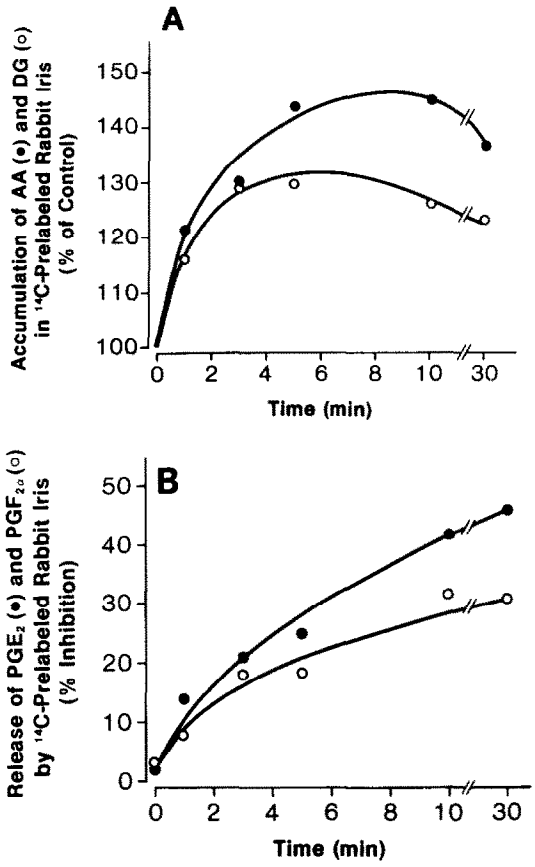

Fig. 2. Time course of the effect of RHC 80267 on DG and AA accumulation (A) and PG release (B) in rabbit iris prelabeled with [^{14}C]AA. Conditions of incubation were the same as described under Table 2 except that the irises were incubated in the absence and presence of RHC (25 μM) for various time intervals as indicated. The tissue was analyzed for DG and AA and the medium was analyzed for PGs as described in the text. The control values (dpm/iris) for AA, DG, PGE_2, and $PGF_{2\alpha}$ were 5023 ± 287, 3927 ± 351, 2321 ± 138, and 1093 ± 78 respectively. The results are the average of two separate experiments.

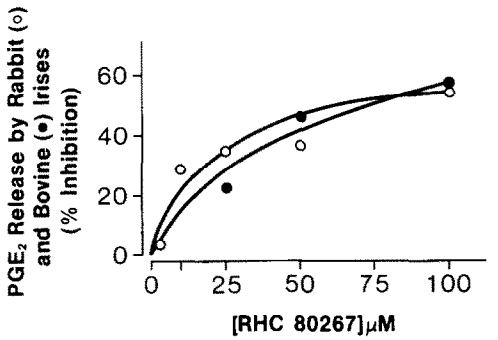

Fig. 3. Effects of RHC 80267 concentration on the basal release of PGE_2 by rabbit (○) and bovine (●) irises. In these experiments one iris from pairs was incubated in 2 ml of Krebs–Ringer buffer in the absence and presence of various concentrations of RHC 80267 for 30 min at 37°. At the end of incubation, PGE_2 in the medium was quantitated by RIA. In the absence of RHC 80267, the basal values for PGE_2 released by rabbit and bovine irises were 2153 ± 101 and 37 ± 3.7 ng/g tissue respectively. The data presented are the means of three separate experiments.

is an inhibitor of DG lipase in the iris, other inhibitors must also be employed in order to evaluate the DG lipase pathway in this tissue, (b) in addition to the DG lipase pathway, other pathways are probably involved in the release of AA in this tissue, and (c) RHC 80267 is an inhibitor of the cyclooxygenase reaction.

Time course of the effect of RHC 80267 *on accumulation of DG and AA and on release of PG in rabbit iris prelabeled with* [^{14}C]*AA.* At a 25 μM concentration of the drug, accumulation of DG and AA increased with time of incubation up to 5 min then leveled off between 5 and 10 min, and declined markedly thereafter (Fig. 2A). Thus, at 5 min the increase in DG and AA accumulation by the drug was 31 and 42%, respectively, and at 30 min this declined to 25 and 36% respectively. In contrast, inhibition of PG synthesis by the drug was time dependent (Fig. 2B). Thus, at 5 min the inhibition of the synthesis of PGE_2 and $PGF_{2\alpha}$ by the drug was 21 and 16%, respectively, and at 30 min it was 46 and 31% respectively. Again these data may suggest that RHC 80267 is an inhibitor of DG lipase in the iris, that various pathways are probably involved in the release of AA, and that this drug is an inhibitor of the cyclooxygenase reaction.

Effect of RHC 80267 *on the basal release of* PGE_2 *by rabbit and bovine irises and by bovine iris microsomes.* Further support for the finding that RHC 80267 is an inhibitor of PG synthesis in the iris came from the data given in Figs. 3 and 4. Thus, the drug inhibited the basal release of PGE_2 by rabbit and bovine irises (Fig. 3) and by bovine iris microsomes (Fig. 4) in a dose-dependent manner. In the presence of 25 μM of the drug, the inhibition of PGE_2 release

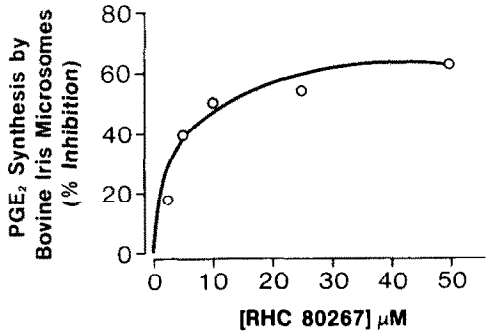

Fig. 4. Effect of RHC 80267 concentration on PGE_2 synthesis by bovine iris microsomes. Bovine iris microsomes, equivalent to 0.25 mg protein, were incubated in 1 ml of 0.1 M phosphate buffer, pH 7.8, that contained 1 μM AA in the absence and presence of various concentrations of RHC 80267, as indicated, for 15 min at 37°. At the end of incubation, PGE_2 was extracted and analyzed by means of RIA. In the absence of RHC 80267, the basal value for PGE_2 released by bovine iris microsomes was 4.78 ± 0.4 ng/mg protein. Results are given as means from three determinations. The data points agreed within 0–5%.

by rabbit and bovine irises was 35 and 27%, respectively, and at 50 μM it was 45 and 41% respectively. Maximal inhibition (60%) of PGE_2 synthesis by bovine iris microsomes was observed at a 25 μM concentration of the drug, and this leveled off between 30 and 50 μM (Fig. 4).

DISCUSSION

A number of pathways for AA release from phospholipids have been proposed. These include: (a) direct deacylation by phospholipase A_2 [2,24–26], (b) degradation of PI by a specific phospholipase C to DG; AA is then released from the latter by the DG/monoacylglycerol pathway [6–8], and (c) degradation of PI by phospholipase C into DG; the latter is then converted into PA which, in turn, is deacylated by a PA specific phospholipase A_2 to release AA [27, 28]. When iris muscle was incubated for 30 min at 37°, there was a significant loss of AA from the major AA-containing phospholipids. These changes have been determined in the present work by GLC (Table 1), and previously similar changes were demonstrated in iris muscle prelabeled with [^{14}C]AA [5]. Addition of RHC 80267 to iris muscle prelabeled with [^{14}C]AA resulted in an increase in the labeling of glycerolipids (Table 2). Since the iris DG lipase, as that of platelets [9–11], were inhibited by the drug (Table 3), we can speculate that the inhibition of this enzyme could lead to the accumulation of [^{14}C]DG, and that the [^{14}C]DG is then converted into PC, PE, TG, PA and PI to increase their labeling. In support of this argument are the data obtained from both the dose–response (Fig. 1A) and time–course (Fig. 2A) studies which showed accumulation of [^{14}C]DG in the drug-treated iris. However, in spite of these findings, and the fact that this drug is a potent inhibitor of DG lipase (Table 3, [9–11]), we observed an increase in the release of AA by the drug (Figs. 1A and 2A). This observation could be explained, in part, by the finding that the drug also inhibited the synthesis of PGs (Figs. 1B, 2B, 3 and 4). However, since only part of the AA accumulated by the drug could come from inhibition of AA conversion into PGs, we have to conclude that part of the released AA must come from the phospholipase A_2 pathway. We have experimental evidence which suggests the formation of lyso PI (S. Y. K. Yousufzai and A. A. Abdel-Latif, unpublished observations), and thus PI, PC and PE could serve as a potential source for AA release in the iris. Although we have no experimental evidence for the presence of a phospholipase A_2 against PA in the iris, PA is attacked by the iris microsomal PA phosphohydrolase [23] to form DG (Table 3); the latter is then metabolized to liberate AA. Chau and Tai [10], working with platelets, reported that RHC 80267 prevents the formation of the transient 2-monoacylglycerol, indicating that the drug inhibits DG lipase in the intact cells. In the same experiments the drug did not inhibit AA release, implying that the DG lipase pathway is not required for AA release. They also found DG accumulation to be similar in control and drug-treated platelets. In contrast, Bross *et al.* [11] reported that RHC 80267 has little effect on the AA metabolism of intact human platelets, and thus concluded that this drug cannot be used to evaluate the DG lipase pathway in intact platelets. The mechanism of action of RHC 80267 on phospholipid metabolism is distinct from that of cationic amphiphilic drugs in that in the latter the overall effects are to stimulate the *de novo* synthesis of CDP-diacylglycerol and the acid phospholipids, PA, the phosphoinositides, and cardiolipin and to depress the synthesis of TG, PC, and PE [15,29–32]. It has been suggested that the rate of conversion to DG, by PA phosphohydrolase, is inhibited by cationic amphiphilic drugs such as propranolol [15, 23, 30–32]. RHC 80267 had no effect on PA-phosphohydrolase and its effect on TG lipase has not been investigated. Rittenhouse-Simmons [33] showed that indomethacin, which at high level doses inhibits DG lipase, causes DG to accumulate to levels five times those of control platelets. The findings in the present work that the microsomal DG lipase was inhibited by the drug and that DG accumulated in the drug-treated tissue suggest to us that this enzyme was inhibited by RHC 80267 in the iris. However, the findings that this drug increased the accumulation of AA and the labeling of glycerolipids and that, in addition, it was an inhibitor of the cyclooxygenase pathway lead us to conclude that data obtained from studies in which this drug was used to investigate the mechanism of AA release from membrane phosphoinositides should be interpreted with caution.

Addendum—After this paper was submitted for publication, a report appeared on the inhibition of AA metabolism in human platelets by RHC 80267 [34]. These authors concluded that RHC 80267, because of its lack of specificity at concentrations needed to inhibit DG lipase, is an unsuitable inhibitor for studying the release of AA in intact human platelets.

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