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Diacylglycerol breakdown in plasma membranes of bovine chromaffin cells is a two-step mechanism mediated by a diacylglycerol lipase and a monoacylglycerol lipase

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The recently identified diacylglycerol lipase activity in membranes of chromaffin cells from bovine adrenal medulla [24] is now shown to consist of two enzymes working in series. First the predominantly saturated fatty acid in the *sn*-1-position is split by a diacylglycerol lipase (glycerol ester hydrolase, EC 3.1.1.34). Subsequently the resulting *sn*-2-monoacylglycerol is split by a monoacylglycerol lipase (glycerol-monoester acylhydrolase, EC 3.1.1.23) which prefers *sn*-2-arachidonoyl-monoacylglycerol to *sn*-2-palmitoyl-monoacylglycerol. At pH 4.0 only the diacylglycerol lipase is active, whereas the monoacylglycerol lipase is irreversibly inactivated. At pH 6.0 both enzymes are active. Pretreatment of the membranes at pH 10 leads to the selective inactivation of the diacylglycerol lipase. Both enzymes are Ca^{2+}- and calmodulin-independent and both are partially inhibited by *p*-bromophenacyl bromide, however, only at relatively high concentrations of the inhibitor. Chlorpromazine inhibits the diacylglycerol lipase to about the same extent as *p*-bromophenacyl bromide but the monoacylglycerol lipase is less sensitive. The specific diacylglycerol lipase inhibitor RHC 80267 (1,6-di(*O*-(carbamoyl)cyclohexanone oxime)hexane) only interacts with the first step, i.e. the diacylglycerol lipase.

Introduction

The exocytosis process in most of the secretory cells by signal release coupling has been shown to depend in the initiation of the phosphoinositol cycle (PI cycle) [1–9]. Subsequently the protein kinase C is activated by the resulting diacylglycerol and the cytosolic [Ca^{+2}] is mobilised by inositol 1,4,5-trisphosphate (IP_3) and in some cases by an increased influx of external Ca^{2+} by voltage or receptor-gated channels or by inhibition of the Ca^{2+}-pump [10,11]. Parallel to this sequence of events, many studies in various cell types have revealed the generation of free arachidonic acid during cell stimulation [12,13], its derivatization to prostanoids and to lipoxygenase products [14–16]. It is not clear, however, whether arachidonic acid and the eicosanoids are absolutely essential for the release process or whether this process parallels the signal-release coupling without influencing it. In chromaffin medullary cells Sasakawa [17,18] and Nishibe [19] have shown that BW 755C (3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline), a lipoxygenase inhibitor, inhibits catecholamine release from these cells, although internal [Ca^{2+}] is increased and protein kinase C is activated after cell stimulation.Both authors postulate that lipoxygenase products may

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be involved in the fusion process of the granules with the plasma membrane, only then leading to exocytosis.

In chromaffin cells, as in many other cell types, it was generally assumed that phospholipase A_2 acting on membrane phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine) was the reason for arachidonic acid generation [7,20,21]. In platelets and other cells such an enzyme was in fact identified [22,23], but no direct evidence was demonstrated for the chromaffin cell. However, we have observed recently that a diacylglycerol lipase is present in plasma membranes of chromaffin cells which acts on diacylglycerol generated by phospholipase C action on PI [24]. We now have characterized this activity and can show that the stimulation is very similar to that in platelets, namely that also in this cell type two enzymes, a diacylglycerol lipase and a monoacylglycerol lipase, are working in series.

Materials and Methods

Materials

Radiolabelled 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol and 1-palmitoyl-2-[1^{14}C]palmitoylphosphatidylcholine were purchased from Amersham International, U.K. Unlabelled diacylglycerol, arachidonic acid, phospholipase C (form *Clostridium perfringens*, type I), chlorpromazine and *p*-bromophenacyl bromide were products of Sigma, St. Louis, MO, U.S.A. RHC 80267 was a generous gift of Rorer Group Inc., Fort Washington, U.S.A.

Membrane preparation

Bovine adrenal glands were removed at the local slaughterhouse and immediately cooled on ice. Plasma membranes of the adrenal medulla were prepared as described previously [24], following essentially the method of Wilson and Kirshner [25].

Preparation of labeled 1-palmitoyl-2-[1-^{14}C]palmitoyl-sn-glycerol

Radiolabelled diacylglycerol was prepared according to Khoo et al. [26]. 2 μCi (33 nmol) of 1-palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine were dried under N_2 and dissolved in 5 ml of diethyl ether. 5 ml of 100 mM Tris-HCl buffer (pH 7.4) and 5 mM $CaCl_2$ were added and the tube was shaken for 1 h at ambient temperature with 200 μl of phospholipase C (from *C. perfringens*, type I, in Tris-HCl buffer (pH 7.4), 1.25 units/ml). Completeness of the reaction was checked by thin-layer chromatography on silica gel plates developed in hexane/diisopropyl ether/acetic acid (60 : 40 : 4, v/v).

Lipase activities

Radiolabelled substrates (20 nCi = 0.33 nmol per sample) were added to the tubes as solutions in hexane and the solvent was evaporated under N_2. The reaction mixture (100 μl) contained isolated plasma membranes (70–80 μg of membrane proteins) and buffer of differed pH. The buffers were prepared according to Handbook of Biochemistry [26], i.e. pH 2–3: glycine-HCl; pH 4–5: acetate; pH 6–7: Tris-maleate; pH 8–9: Tris-HCl and pH 10–12: glycine-NaOH. The inhibitors were added as 5 μl solutions in water (chlorpromazine), acetone (*p*-bromophenacyl bromide) and dimethylsulfoxide (RHC 80267). Controls with the same volume of solvents were also performed (inhibitor concentration = 0 μM). The samples were sonicated in a sonication bath for 30 s and incubated for variable times at 37 °C in a shaking water bath. The reaction was terminated with 100 μl of ethanol, containing 2% acetic acid, arachidonate, diacylglycerol and monoacylglycerol as carriers (10 μg of each) according to Ballou and Cheung [22].

Analytical procedure

After mixing, 50-μl aliquots of each sample were transferred to a counting vial and another 50 μl were applied to a 20 × 20 cm Silica gel 60 thin-layer chromatography plate (Merck, Darmstadt, F.R.G.), which subsequently was developed in a solvent system of hexane/diisopropyl ether/acetic acid (60 : 40 : 4, v/v) to separate diacylglycerol, monoacylglycerol and free fatty acid. After development of the plates and coloration in iodine vapour, the areas corresponding to these lipids were scraped off and transferred into scintillation vials. Radioactivities in the vials were measured after adding 4 ml of scintillation fluid containing PPO/POPOP (2,5-diphenyloxazole/1,4-

bis-(5-phenyl-2-oxazolyl)benzene): 20 g PPO, 0.8 g POPOP in 4000 ml toluene, 3407 ml Triton X-100 and 148 ml acetic acid. Results are given in percent of total radioactivity found in the three analysed lipids. All the experiments were made in duplicate or triplicate. The results were reproduced at least once and varied in the range of ±10%.

Results

In a previous publication [24] we described the presence of diacylglycerol lipase in plasma membranes in bovine adrenal chromaffin cells. This enzyme liberates arachidonic acid from diacylglycerol originating from PI, which was previously split by a phospholipase C. The aim of the present work was to characterize this diacylglycerol lipase. Lipase activities were measured as a function of the pH value of the reaction mixture (Fig. 1). At pH 5.0 only radiolabelled monoacylglycerol is present as a reaction product, whereas at higher pH mainly radiolabelled arachidonate is formed. Fig. 2 shows the time-dependent formation of monoacylglycerol and arachidonate at pH 4.0 and 6.0, respectively. From these results it can be concluded that at low pH a diacylglycerol lipase is active. The lipase exclusively releases the fatty acid in position 1 of the diacylglycerol as only arachidonate in position 2 is radioactively labelled. At higher pH,however, the level of monoacylglycerol remains low, whereas free arachidonate is released. There are three possibilities to interpret this result:

(1) There is a diacylglycerol lipase with two different pH optima. At pH 4.0 it shows a preference for position 1 of the diacylglycerol, whereas at pH 6.0 it preferentially splits off the fatty acid in position 2.

(2) There are two different diacylglycerol lipases with specificities for position 1 or 2. They have a different pH optimum.

(3) There are two enzymes, a diacylglycerol lipase with a preference for position 1, and a monoacylglycerol lipase, which splits the monoacylglycerol formed by the first enzyme. At physiological pH this monoacylglycerol lipase is working faster than the diacylglycerol lipase.

As we presumed that this third hypothesis was most probable, more experiments were made to verify this assumption. It was investigated whether the monoacylglycerol-lipase activity, which was absent at pH 4.0, could be restored by changing the incubation medium from pH 4.0 to pH 6.0 (Fig. 3). After 60 min incubation at pH 4.0 about 60% of the diacylglycerol was hydrolysed to monoacylglycerol. After washing with buffer pH 6.0 the membranes were reincubated at 37 °C. The level of monoacylglycerol remained constant indicating that the monoacylglycerol-lipase activity was destroyed irreversibly at pH 4.0. When new membranes were added to the samples, monoacylglycerol was split and arachidonic acid was liberated. This confirmed the presence of monoacylglycerol-lipase activity in the membrane preparation. The monoacylglycerol lipase is labile

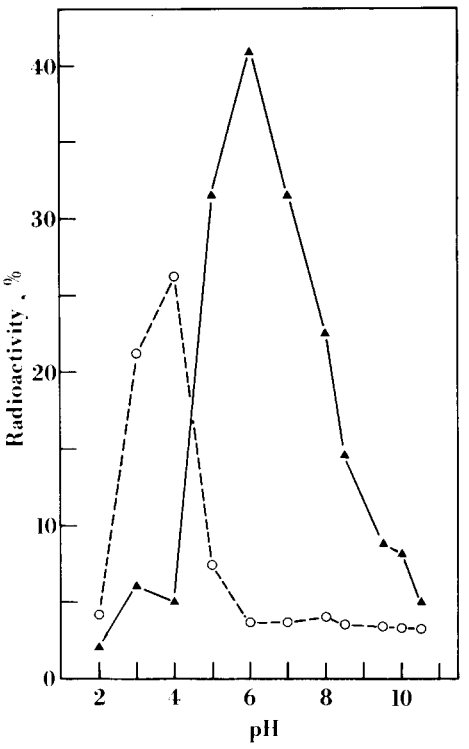

Fig. 1. pH-dependence of diacylglycerol-degradation. Membranes (75 μg protein) were incubated with 1-stearoyl-2[1-^{14}C]arachidonoyl-diacylglycerol for 60 min at 37 °C in presence of buffers of different pH values, which were prepared as described in Methods. pH was measured in the incubation mixture. The samples were stopped and analysed as described in Methods (monoacylglycerol, O - - - O; free arachidonic acid, ▲——▲). The results are representative for two experiments.

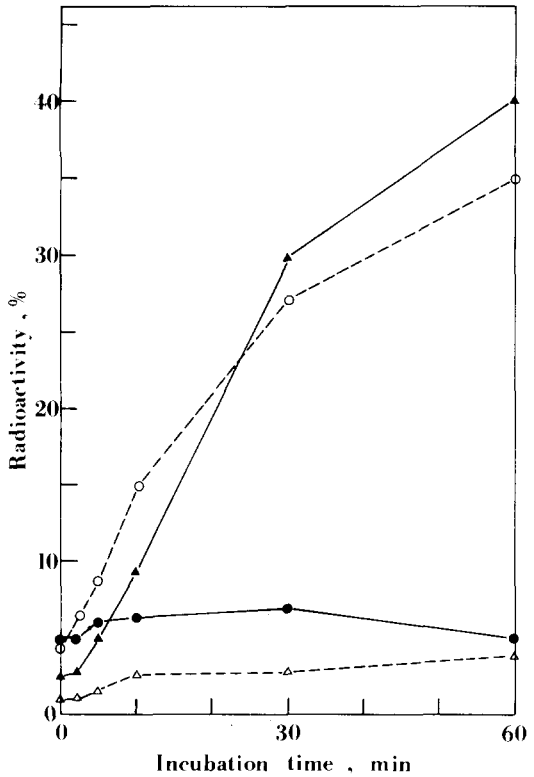

Fig. 2. Time-dependent hydrolysis of diacylglycerol at pH 6.0 and pH 4.0. Membranes (75 μg protein) were incubated at 37°C in presence of 1-stearoyl-2-[1-^{14}C]arachidonoyl-diacylglycerol at pH 6.0 and at pH 4.0. After different times the reaction was stopped and the samples were analysed as described in Methods (pH 6.0: arachidonic acid, ▲———▲; monoacylglycerol, ●———●. pH 4.0: arachidonic acid, △- - -△; monoacylglycerol, O- - -O). The results are representative for a set of six experiments.

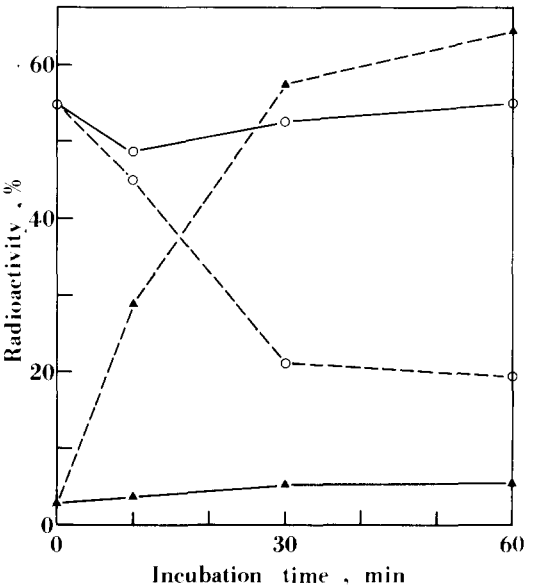

Fig. 3. Monoacylglycerol degradation after preincubation at pH 4.0. Membranes (75 μg protein) were incubated with 1-stearoyl-2-[1-^{14}C]arachidonoyl-diacylglycerol at pH 4.0 for 60 min at 37°C and subsequently washed with buffer of pH 6.0. The samples were reincubated at pH 6.0 either in presence (monoacylglycerol, O- - -O; arachidonic acid, ▲- - -▲) or in absence (monoacylglycerol; O———O; arachidonic acid, ▲———▲) of new membranes (75 μg protein). The reaction was stopped and the samples were analysed as described in Methods. The results are representative for four experiments.

with regard to acidic treatment, whereas the diacylglycerol-lipase activity remains unaffected at pH 4.0.

Following the finding of Mauco et al. [28] in platelets that only the diacylglycerol lipase is sensitive to alkaline treatment, we investigated whether any of the enzyme activities could be influenced by alkaline pretreatment (Fig. 4). Membranes were washed with buffer pH 10.0 and left in this buffer for 60 min in ice. The samples were recovered by centrifugation and washed with pH 6.0 buffer. To ascertain if the washing and centrifugation procedures may injure the enzyme activities, membranes were treated identically with pH 6.0 buffer instead of pH 10.0. After this pretreatment the membranes were incubated with labelled diacylglycerol at 37°C. Fig. 4 demonstrates that after alkaline preincubation there was significantly less arachidonic acid released. As monoacylglycerol was not present (results not shown), it appeared that only diacylglycerol lipase was inhibited and not the monoacylglycerol lipase. To underline this fact, membranes were incubated at pH 4.0 with labelled diacylglycerol for 60 min to generate samples containing labelled monoacylglycerol. After washing and recovering the samples in pH 6.0 buffer, they were reincubated with membranes which were pretreated as described above at pH 10.0, resp. 6.0. Fig. 4 shows that the monoacylglycerol-lipase activity was not lowered by alkaline pretreatment.

These experiments confirmed the hypothesis that two different enzymes are involved in diacylglycerol degradation: a diacylglycerol lipase labile towards alkaline treatment and a monoacylglycerol lipase labile towards acidic treatment.

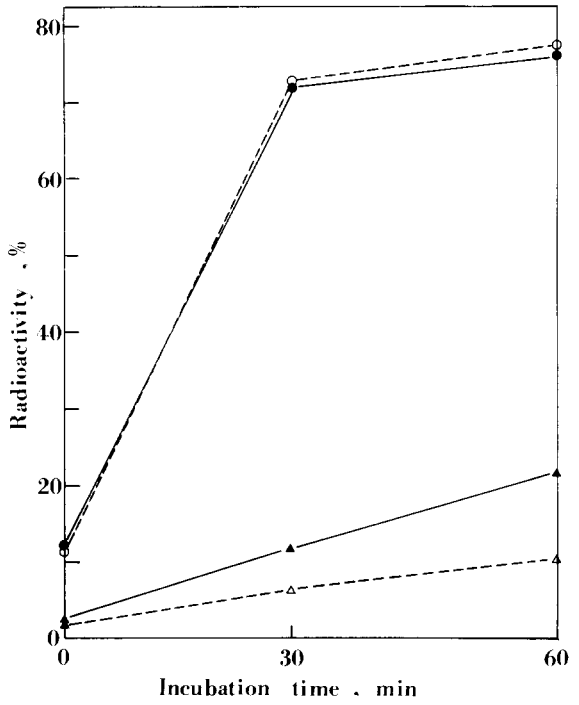

Fig. 4. Sensitivity of diacylglycerol lipase and monoacylglycerol lipase to alkaline treatment. Membranes (75 μg protein) were washed with buffer of pH 10.0. After leaving them in this buffer for 60 min at 0 °C they were centrifuged, washed and taken up in buffer of pH 6.0 for the incubation with 1-stearoyl-2-[1-^{14}C]arachidonyl-diacylglycerol. The release of free arachidonic acid was measured with membranes pretreated at pH 10 (△– – –△) and as a control with membranes pretreated the same way, but with buffer of pH 6.0 (▲———▲). Monoacylglycerol lipase was measured by preincubation at pH 4.0 as described in Fig. 3. After adding new membranes (75 μg protein), which had been pretreated at pH 10.0 (○– – –○), and as a control at pH 6.0 (●———●), the release of arachidonic acid was measured. The results are representative for three experiments.

The Ca^{2+}-dependence of the diacylglycerol lipase was tested by adding EDTA (10 mM) or Ca^{2+} at different concentrations (10 μM–10 mM) in presence and in absence of calmodulin (10 μM). No difference in arachidonic acid liberation could be observed (results not shown).

The two enzymes have further been studied for their sensitivity towards various known inhibitors of lipolysis. By addition of RHC 80267, known as a specific inhibitor of diacylglycerol lipases, the degradation of diacylglycerol was completely inhibited at pH 4 at a concentration of 20 μM (Fig. 5). This means that the diacylglycerol lipase is

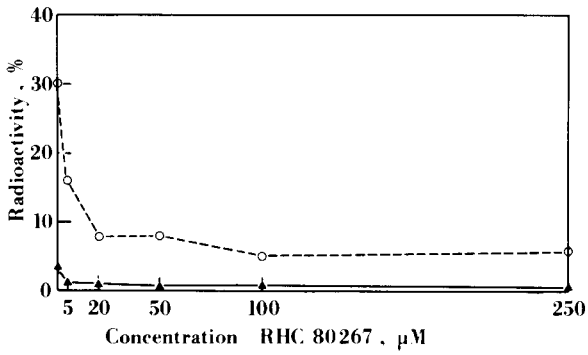

Fig. 5. Inhibition of diacylglycerol lipase by RHC 80267 at pH 4.0. Membranes were incubated with 1-stearoyl-2[1-^{14}C]arachidonoyl-diacylglycerol in presence of the inhibitor RHC 80267 (0–250 μM) for 30 min at 37 °C at pH 4.0. The reaction was stopped and the samples were analysed as described in Methods (monoacylglycerol, ○– – –○; arachidonic acid, ▲———▲). The results are representative for two separate experiments.

inhibited. To see whether the monoacylglycerol lipase was also inactivated by the inhibitor, membranes were preincubated with labelled diacylglycerol at pH 4.0 to generate monoacylglycerol, then washed and restored in buffer pH of 6.0. Non-treated membranes were added together with the inhibitor (20 μM) and incubated for 60 min at 37 °C. Table I shows that monoacylglycerol is disappearing and arachidonic acid is liberated in presence and in absence of the inhibitor. Monoacylglycerol lipase is therefore not or only slightly inhibited by RHC 80267.

Other inhibitors were also tested. Mepacrine

TABLE I

INHIBITION OF MONOACYLGLYCEROL LIPASE BY RHC 80267

Membranes (75 μg) protein were incubated with 1-stearoyl-2-[1-^{14}C]arachidonoyl-diacylglycerol at 37 °C for 60 min at pH 4.0 to generate labelled monoacylglycerol. The samples were washed with buffer of pH 6.0 and reincubated with new membranes (75 μg protein) for 60 min at 37 °C in presence and in absence of RHC 80267 (20 μM) to measure monoacylglycerol-lipase activity. The results are mean values of two separate experiments.

	% monoacyl-glycerol	% arachido-donic acid	% diacyl-glycerol
pH 4, 60 min	67.5	2.3	30.2
pH 6, −RHC 80267	16.4	57.6	26.1
pH 6, +RHC 80267	12.9	52.3	34.7

had no effect on both enzymes (Results not shown), whereas *p*-bromophenacyl bromide and chlorpromazine slightly inhibited the diacylglycerol lipase (Fig. 6). Monoacylglycerol-lipase activity was unaffected by chlorpromazine, whereas *p*-bromophenacyl bromide again showed a slight inhibition, however, only at concentrations of 100–250 μm (Fig. 6).

To examine, whether any of the enzyme has a preference for substrates containing unsaturated fatty acids, the membranes were incubated in presence of two different diacylglycerols: (1) 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol and (b) 1-palmitoyl-2-[1-^{14}C]palmitoyl-diacylglycerol. First the experiment was made at pH 4.0 to test the diacylglycerol lipase. Fig. 7 shows that the substrate containing arachidonic acid is degraded

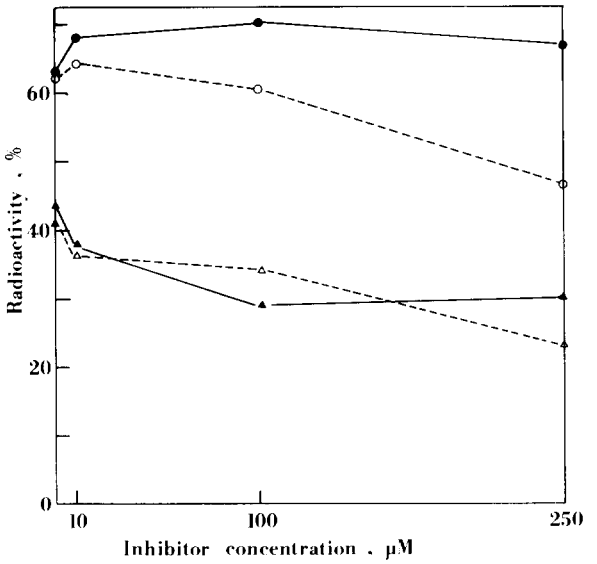

Fig. 6. Inhibition of diacylglycerol lipase and monoacylglycerol lipase by chlorpromazine and *p*-bromophenacyl bromide. Membranes (75 μg protein) were incubated at 37 °C with 1-stearoyl-2-[1-^{14}C]arachidonoyl-diacylglycerol at pH 4.0 in presence of inhibitors (0–250 μM) for 30 min at 37 °C. Release of arachidonic acid from diacylglycerol was measured as described in Methods (chlorpromazine, ▲———▲; *p*-bromophenacyl bromide, △– – –△). For monoacylglycerol lipase the membranes were preincubated at pH 4.0 for 60 min to generate monoacylglycerol and then washed with buffer of pH 6.0. New membranes were added together with the inhibitor and the release of arachidonic acid was determined (chlorpromazine, ●———●; *p*-bromophenacyl bromide, ○– – –○). The results are representative for three separate experiments.

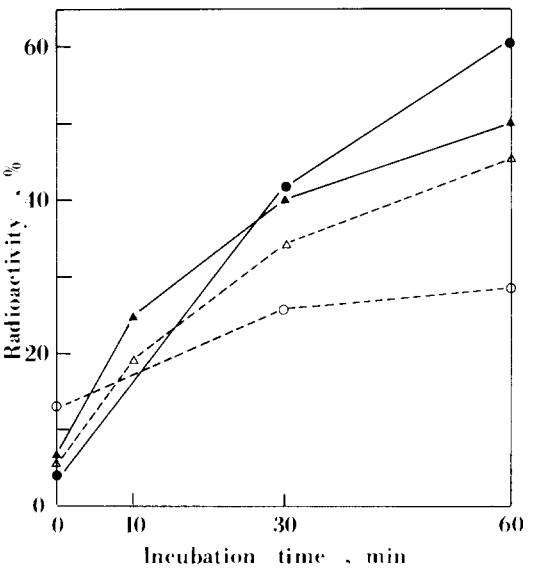

Fig. 7. Substrate specificity of diacylglycerol lipase and monoacylglycerol lipase. Membranes (75 μg protein) were incubated at pH 4.0 with two different substrates: 1-stearoyl-2-[1-^{14}C]-arachidonoyl-*sn*-glycerol and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol. The release of arachidonic acid (▲———▲) and palmitic acid (△– – –△) was measured. For the specificity of the monoacylglycerol lipase the membranes were preincubated at pH 4.0 for 60 min. After washing with buffer of pH 6.0 new membranes were added and the release of arachidonic acid (●———●) and palmitic acid (○– – –○) was measured as described in Methods. The results are representative for four separate experiments.

at almost the same rate as the substrate with saturated fatty acids only. As the fatty acids in the two substrates are different in position 1 as well as in position 2 it is not possible to decide, whether this preference is due to the arachidonic acid in position 2 or to the longer fatty acid in position 1. In a second set of experiments the substrates were first hydrolysed at pH 4.0 to generate labelled monoacylglycerol, then washed and restored in buffer of pH 6.0. New membranes were added to test the monoacylglycerol-lipase activity towards the two different substrates (i.e. *sn*-2-palmitoyl-glycerol and *sn*-2-arachidonoyl-glycerol) (Fig. 7).

There is a clear difference between the two substrates. Whereas the substrate with arachidonic acid is degraded almost completely after 60 min, the second with the saturated fatty acid is only slightly degraded. The monoacylglycerol lipase

shows a preference for 2-arachidonoyl-monoacylglycerol compared to 2-palmitoyl-monoacylglycerol.

Discussion

The first indication of a two-step mechanism in the breakdown of diacylglycerol in chromaffin cells, which previously was thought to be one step, came from the observation of the particular pH-dependence of the two hydrolytic reactions (Fig. 1). The main goal of this work consisted in showing whether in chromaffin cells, similar to platelets [29,30] and rat brain microsomes [31], two enzymes, a diacylglycerol lipase and a monoacylglycerol lipase working in series, or if one acylhydrolase acting on the 1- and 2-position of diacylglycerol with different pH optimum was responsible for the generation of arachidonic acid.

The fact that at pH 4 only the first step, i.e. the deacylation of diacylglycerol in position 1, takes place, without further breakdown of the generated monoacylglycerol (Fig. 2) allowed the study of this first step separately. This is not possible at pH 6 where both reactions take place simultaneously (Fig. 2). At pH 4, by using a diacylglycerol labelled in the fatty acid at the 2-position, the catalytic activity for the second step, i.e. monoacylglycerol breakdown, is irreversibly destroyed. It is thus possible to study this second step independently by first generating the labelled monoacylglycerol at pH 4, changing the pH to 6 and then adding new membranes, or alternately using labelled monoacylglycerol as substrate.

By this experimental procedure we were able to study the two steps of diacylglycerol breakdown separately with regard to their sensitivity to Ca^{2+}, to various inhibitors and for their preferences with respect to the fatty acid composition of the substrates.

Both steps of diacylglycerol deacylation in chromaffin cell membranes are independent on Ca^{2+} and calmodulin. This is in partial disconcordance with various studies of the diacylglycerol lipase in other cell types. In platelets Authi et al. [32] described a membrane bound diacylglycerol lipase which is stimulated by EGTA or reduced glutathion, whereas Mauco et al. [28] find a diacylglycerol lipase in plasma membrane fractions of platelets which is strongly Ca^{2+}-dependent with an optimum at 10^{-3} M [Ca^{2+}] or [Mg^{2+}]. Strosznajder et al. [33] report that a diacylglycerol lipase in subcellular fractions of rat brain is slightly enhanced by Ca^{2+} (1 mM), but that 0.5–4 mM Ca^{2+} in the presence of 10 mM Mg^{2+} inhibited the diacylglycerol kinase. At present it is difficult to speculate about the comparability of these diacylglycerol lipases from different cell types.

Mauco et al. [28] report that only the first step, i.e. diacylglycerol lipase is sensitive towards alkaline treatment of the membranes at pH 10. This is in full agreement with what we found in the plasma membranes of chromaffin cells (Fig. 4). Like Mauco et ai. also we consider these data as supporting the postulate that we are dealing with two distinct enzymes.

Studies in platelets on the specificity of the diacylglycerol lipase with respect to the fatty acid composition of the substrates have shown that diacylglycerol containing arachidonic acid in the 2-position is preferentially hydrolysed [32]. We can fully confirm these findings for the overall reactions in chromaffin cell membranes, however, we can specify that in the first step, i.e. elimination of the saturated fatty acid in the 1-position, no preference can be observed (Fig. 7), whereas by the monoacylglycerol lipase step very clearly arachidonic acid containing monoacylglycerol is preferred to 2-palmitoyl-monoacylglycerol (Fig. 7). It can not be excluded that the different rates of fatty acid liberation from the two substrates are due to a better accessibility of the arachidonic acid containing monoacylglycerol in the membrane, and not to the specificity of the enzyme. To prove a clear preference of the enzyme for arachidonic acid, substrates containing various polyunsaturated and monounsaturated fatty acids at the *sn*-2-position will have to be used, as it was done in platelets [32]. We also consider this as a strong argument in favour of the presence of two distinct enzymes.

Further support of the two-enzyme hypothesis comes from the independent study of the two steps by using various inhibitors.

Sutherland and Amin [34] first described RHC 80267 (1,6-di(*O*-(carbamoyl)cyclohexanone oxime)hexane) as a specific inhibitor of canine platelet diacylglycerol lipase. We can confirm this

inhibitory effect of RHC 80267 in chromaffin cell membranes, and in addition we now can specify that only the first step, i.e. the diacylglycerol lipase, is sensitive to this compound, whereas the monoacylglycerol lipase remains practically unaffected at 20 μM RHC 80267 (Fig. 4 and Table I). According to Oglesby and Gorman [35], this inhibitor was found ineffective in intact human platelets at 20 μM. However at higher concentration (> 200 μm) diacylglycerol lipase was impaired, as well as the phospholipases C and A_2. Possible explanations for the discrepancies existing between various authors concerning the specificity and the dose response of RHC 80267 have been proposed [36–38]. This may be a warning that diacylglycerol lipases from different cell types, although similar, may show specific differences.

p-Bromophenacyl bromide, a strong and well known inhibitor for a number of hydrolytic enzymes, also interacts with the diacylglycerol and monoacylglycerol-lipase system, but only at relatively elevated concentrations and not as discriminately as RHC 80267. Even at 100 μM and up to 250 μM an inhibition of maximally 25% is observed for the diacylglycerol lipase (Fig. 6), whereas the monoacylglycerol lipase is hardly affected up to 100 μM inhibitor concentration (Fig. 6) and only at 250 μM a reduction of about 20% is shown. Chlorpromazine, which is supposed to interfere with the Ca^{2+}-binding of the Ca^{2+}-dependent phospholipase A_2 shows a weak effect on the diacylglycerol lipase comparable to the effect of *p*-bromophenacyl bromide (Fig. 6), but it does not influence at all the monoacylglycerol lipase (Fig. 6).

All together our data obtained for the two steps of diacylglycerol deacylation strongly support the existence of two separate enzymes, a diacylglycerol and a monoacylglycerol lipase, as was proposed for platelets [28,32]. In fact, the situation in platelets is very similar. The properties of the two enzymes appear almost identical in chromaffin cells and in platelets.

Preliminary experiments have shown that by differential detergent solubilization and subsequent chromatography a partial separation is possible. Further work on the isolation of the two enzymes is in progress in our and other laboratories [36].

The question whether, beside the diacylglycerol and the monoacylglycerol lipase system in chromaffin cells, a phospholipase A_2 may also be the generating agent of free arachidonic acid is still open. The reported release of labelled arachidonic acid after preincubation of chromaffin cells and nicotinic stimulation [7,20,21] may indeed be an indication for the presence of a Ca^{2+}-dependent phospholipase A_2. From the data of these authors, however, it cannot be excluded that the stepwise action of phospholipase C, diacylglycerol lipase and monoacylglycerol lipase may be the only source of the generated arachidonic acid.

Further studies are necessary to demonstrate that in addition to the two-step enzyme system acting on initial phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate another, e.g. phospholipase A_2 acting on other phospholipids, e.g. phosphatidylcholine, is present in plasma membranes of chromaffin cells. The possibility that the phospholipase C-diacylglycerol lipase-monoacylglycerol lipase system may be sufficient, at least in chromaffin cells, for arachidonic acid generation, as has recently been proposed by Moscat et al. [39] for macrophage stimulation, has to be left open. In platelets stimulated with high concentrations of thrombin, the main part of arachidonic acid generation (80%) is due to phospholipase A_2 acting predominantly on phosphatidylcholine [40], whereas lower concentrations of the agonist seem to liberate preferentially arachidonic acid from phosphatidylinositol [41]. It will be important to establish the eventual role of arachidonic acid and the eicosanoids in the release mechanism, e.g. the fusion of granular membrane with the plasmamembrane, as supposed by De Lisle and Williams [42].

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