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INACTIVATION AND SOLUBILIZATION OF OPIATE RECEPTORS BY PHOSPHOLIPASES A_2*

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(1) As previously shown, stereospecific binding of opiates to membrane bound receptor is inhibited by treatment with small amounts of phospholipase A_2 from *Vipera russelli*. This effect is quantified and compared with the enzymes from the venoms of *Naja naja siamensis*, *Apis mellifica* and from porcine pancreas. All enzymes are equally effective. The inhibition is due to partial phospholipid hydrolysis leading to inactivation of membrane-bound receptor. (2) Bee venom phospholipase A_2 together with the synergistically acting peptide, melittin, causes receptor solubilization: up to 80% of preformed receptor-ligand complex can be solubilized in this manner. (3) Lysophosphatidylcholine, a product of phospholipid hydrolysis, solubilizes preformed receptor-ligand complex to a similar extent. Several other detergents were tested for their ability to solubilize receptor-ligand complex. Digitonin appears to be most effective in solubilizing such a complex.

Introduction

Opiates exert their actions on target cells through specific binding to membrane proteins called opiate receptors [2–4]. Membrane lipids appear to be an essential part of these receptors. Treatment of brain membranes with phospholipase A_2 abolishes completely stereospecific opiate binding [5–8].

Until recently, attempts to solubilize active opiate receptors from rat brain membranes have met with little success. Therefore, studies on the conditions of solubilization are commonly performed with membranes prelabelled with [^{3}H]opiates. The most effective detergent for this purpose is Brij 36 T [9] which has been used in other laboratories [10,11]. Sodium cholate has recently been used successfully [12].

We report here a study of the inactivating effect of several kinds of phospholipases of the A_2 type on the opiate receptor present in rat brain membrane. We show also that one of the enzymes studied, bee venom phospholipase, can solubilize the receptor-opiate complex when used together with the synergistically acting bee venom peptide melittin [13].

Materials and Methods

Materials

[^{3}H]Etorphine (33 Ci/mmol) and [^{3}H]diprenorphine (19.2 Ci/mmol) were obtained from the National Institute on Drug Abuse and were made up as aqueous stock solutions (10^{-7} M). [^{3}H]Diprenorphine (6.7 Ci/mmol) was also ob-

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tained from Amersham. Digitonin (Lot 300034), Zwittergent 3-14 (Lot 810055) and octyl-β-D-glucoside (Lot 494459) were from Calbiochem. Brij 36 T was from Atlas Chemical Industries. Triton X-114 was from Rohm and Haas (Lot 6-1583) and Triton X-100 (Lot 1-8878), sodium deoxycholate (Lot 56C-0041) and L-α-lysophosphatidylcholine (Lot 88C-8380) were from Sigma. Bovine serum albumin (fatty acid free, lot 24) was from Miles. Egg yolk phosphatidylcholine was from Merck, Darmstadt, F.R.G. Bee venom (*Apis mellifica*) was from Sigma (grade 1, lot 69C-0243). Melittin was isolated from bee venom by gel filtration and CM-cellulose chromatography [14]. Phospholipase A_2 was purified from bee venom by the method of Shipolini et al. [15] omitting the last two ion-exchange steps; specific activity was 2520 units/mg. It was also purified from the venom of *Naja naja siamensis* by DEAE-cellulose chromatography and preparative isoelectric focusing [16]. The material with a p*I* of 4.9 was used (379 units/mg). Phospholipase A_2 from *Vipera russelli* was from Sigma (780 units/mg) and that from porcine pancreas (2040 units/mg) as well as its proenzyme [17] were gifts from Dr. De Haas (State University of Utrecht, The Netherlands).

Methods

Rat brain membranes. Adult rats (D/A strain) were killed by decapitation and brains without cerebellum were homogenized with a glass-teflon homogenizer in 6 parts of ice-cold 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 (buffer A). After centrifugation (30000 × *g*, 10 min, 4°C), the pellet was rehomogenized with a glass-glass homogenizer in 6 parts of ice-cold buffer A and centrifuged as described above. This wash-cycle was repeated once and the final pellet suspended in 6 parts of ice-cold buffer A containing 0.32 M sucrose. The protein concentration (determined by the method of Lowry et al. [18] in the presence of 2% sodium dodecyl sulfate using bovine serum albumin as standard) was 9.5–10 mg/ml and the maximal specific binding of [^{3}H]diprenorphine was 0.4–0.5 pmol/mg protein. Membrane suspensions were stored at −70°C for periods of up to four months.

Phospholipase Assay. The activity was determined as described by De Haas et al. [17] but using, as substrate, 5 ml of an ultrasonicated suspension of egg yolk phosphatidylcholine (4 mM) and 2.5 mM sodium deoxycholate in 6 mM $CaCl_2$. Titration was carried out at 37°C and pH 8.5 with NaOH (0.1 M) using a Radiometer Autotitrator 11. One unit of phospholipase activity corresponds to the hydrolysis of 1 μmol substrate per min.

Inactivation experiments. Rat brain membranes were diluted into buffer A to a final protein concentration of 0.7–1.2 mg/ml. They were treated with phospholipases or L-α-lysophosphatidylcholine with or without calcium chloride (5 mM) at 37°C for 10 min. When $CaCl_2$ was present, the reaction was terminated by the addition of 0.5 M K-EDTA, pH 7.4 to a final concentration of 0.1 M. Treatment with bovine serum albumin was at 0°C for 30 min. Opiate binding was determined as described under 'Filtration assay'.

Solubilization experiments. Rat brain membranes were incubated at 37°C for 15 min in buffer A at a protein concentration of 0.9–1.2 mg/ml with [^{3}H]diprenorphine (1.2–2.0 nM) or [^{3}H]etorphine (0.7–0.9 nM). Incubation for non-specific binding was performed in parallel in the presence of 10^{-6} M levorphanol. After cooling on ice, 0.6-ml aliquots of the prelabelled membranes were mixed 1 : 1 with solubilizing agents in buffer A at 0°C for 30 min unless otherwise indicated. The suspension was centrifuged at 4°C (30000 × *g* for 10 min and in some cases 100000 × *g* for 60 min). An aliquot (0.9 ml) of the supernatant was used for gel filtration assay and another aliquot (0.05–0.1 ml) for protein determination [18]. Radioactivity remaining in the pellet was assayed after solubilizing at 50°C for 120 min with 0.5 ml Protosol (New England Nuclear) in 2 ml of Econofluor (NEN).

Filtration assay. A slight modification of a method described earlier [19] was used. Suspensions of treated or untreated membrane fragments were diluted in buffer A to a protein concentration of 0.6–1.2 mg/ml. Incubation with [^{3}H]etorphine or [^{3}H]diprenorphine (0.5–1.5 nM) was for 15 min at 37°C. Non-specific binding was measured under identical conditions but in the presence of 10^{-6} M levorphanol. After cooling on ice for 5 min, the suspension was filtered through a Whatman GF-C filter and washed twice with 4 ml ice-cold buffer A. The filter was dried and radioactivity determined using 2 ml Econofluor (NEN) in poly-

propylene tubes (0.9 × 5 cm).

Gel filtration assay. All steps were performed at 4°C. The 30000 × *g* or 100000 × *g* supernatants (0.9 ml) of solubilized [^{3}H]opiate complex were applied to columns (0.8 × 20 cm) of Sephadex G-50 fine covered with 0.5 cm of Sephadex G-10. The columns were washed with 2 ml of buffer A. The material in the void volume was eluted with 3 ml of buffer A. The unbound radioactivity was eluted with 8 ml of buffer A. Aliquots (1 ml) of the material in the void volume and of the unbound radioactivity were mixed with 8 ml of Biofluor (NEN) and counted. Recovery of radioactivity was 92–100%. The columns were calibrated with Blue dextran and $Na_2{}^{35}SO_4$ (Fig. 3).

Results

1. Inactivation by phospholipases A_2 of opiate binding to rat brain membranes

Phospholipases of A_2 specificity from four different sources were purified according to published procedures and their activities were determined using phosphatidylcholine as substrate [17]. Rat brain membranes were incubated with these enzymes under different conditions, the reaction was stopped by adding EDTA and membrane bound opiate receptor was assayed using [^{3}H]diprenorphine as opiate receptor ligand.

Fig. 1 illustrates the results obtained in a quantitative manner. The four enzymes inhibited opiate binding at very similar activities. Inhibition was complete at enzyme concentrations of about 1 unit/ml. 50% inhibition was obtained at concentrations of 0.03–0.08 units/ml. Depending on the specific activity of the enzyme used, this represents 20–120 ng enzyme/ml. All enzymes showed full activity with respect to both phosphatidylcholine hydrolysis [17] and inhibition of opiate binding after heating them at 100°C for 10 min. On the other hand, no such activity was observed when the phospholipase treatment was carried out in the absence of $CaCl_2$ (Fig. 1). In another control experiment (not shown), the effect of the porcine pancreatic proenzyme (1 μg/ml) was tested. As reported earlier [8], no inhibition of opiate binding occurred with this preparation. Participation of membrane-bound endogenous phospholipases was excluded by experiments in which the membranes were incubated for prolonged periods of time (1 h, 37°C) in the presence of 5 mM $CaCl_2$.

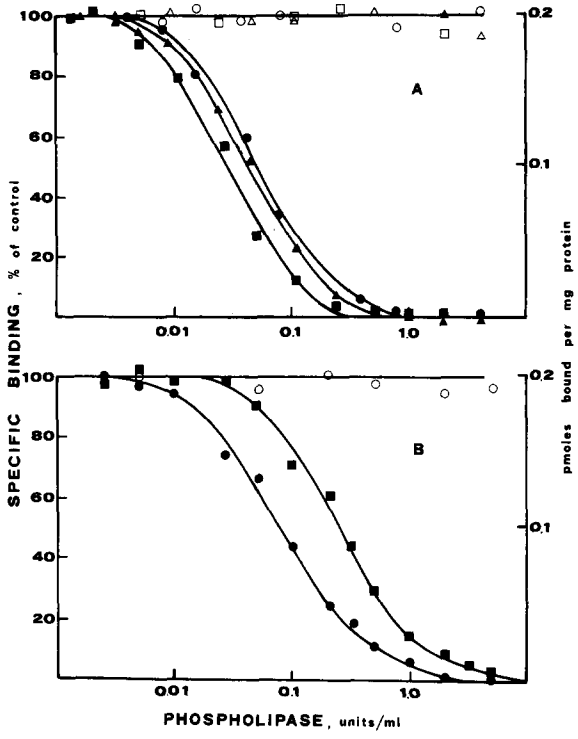

Fig. 1. Inactivation of specific opiate binding by treatment of rat brain membranes with phospholipases of A_2 specificity. A suspension of membranes (1.05 mg protein/ml) was treated for 10 min at 37°C with different concentrations of each of the enzymes tested. The reaction was stopped by addition of EDTA. Binding was determined using [^{3}H]diprenorphine (0.85 nM) and the filtration assay. (A) The phospholipases were from the venoms of *Vipera russelli* (780 units/mg; ○, ●) and *Naja naja siamensis* (379 units/mg; △, ▲) and from the porcine pancreas (2040 units/mg; □, ■). (B) The phospholipase was from the venom of *Apis mellifica* (2520 units/mg; ○, ●). The shift is due to further treatment with bovine serum albumin (1 mg/ml) at 0°C for 30 min (■). Open symbols refer to experiments without $CaCl_2$.

As previously reported [6,8], when phospholipase-treated membranes were exposed (30 min, 0°C) to defatted bovine serum albumin (1 mg/ml) before assaying for opiate binding, a partial recovery of binding was observed. This effect is shown in Fig. 1B using the enzyme from bee venom. Under these conditions, inactivation of opiate binding required three times as much enzyme.

TABLE I

SOLUBILIZATION OF [^{3}H]DIPRENORPHINE RECEPTOR COMPLEX BY A VARIETY OF DETERGENTS

Rat brain membranes (1.17 mg protein/ml) were incubated with [^{3}H]diprenorphine (1.15–1.87 nM) for 15 min at 37°C in the presence or absence of 10^{-6} M levorphanol. This prelabelled preparation was further treated for 30 min on ice with detergent. After centrifugation, gel filtration and protein assay was performed with the supernatant (Fig. 3) and the pellets were used for determination of specifically bound ligand (see Methods). Results are averages of two to three independent experiments.

Detergent		Stereospecific binding (% of control) [a]		Protein solubilized (% of control) [b]
Type	mg/ml	Membrane bound	Solubilized	
None [a]	–	103	0	2
Brij 36 T	2	21	49	39
Triton X-100	1	17	30	38
Triton X-114	1	12	32	37
Sodium deoxycholate	2	53	36	36
Digitonin	0.1	81	3	11
Digitonin	1	65	31	30
Digitonin	5	19	73	35
Lysophosphatidylcholine	0.01	108	0	n.d.
Lysophosphatidylcholine	0.1	22	0	13
Lysophosphatidylcholine	2	0	12	22
Lysophosphatidylcholine	8	0	70	41
Octyl glucoside	2	104	0	17
Octyl glucoside	20	4	0.5	72
Zwittergent	1	19	14	31
Zwittergent	10	5	18	78

[a] Percentage relative to the amount of membrane bound receptor labelled in parallel and tested by filtration assay.
[b] Percentage relative to amount of protein solubilized by sodium dodecyl sulfate (2%).

When L-α-lysophosphatidylcholine was added to the membranes prior to labelling with opiate, inactivation was observed as well (Fig. 2). The concentrations of lysophosphatidylcholine required for receptor inactivation were lower than those required for solubilizing the prelabelled receptor (Table I).

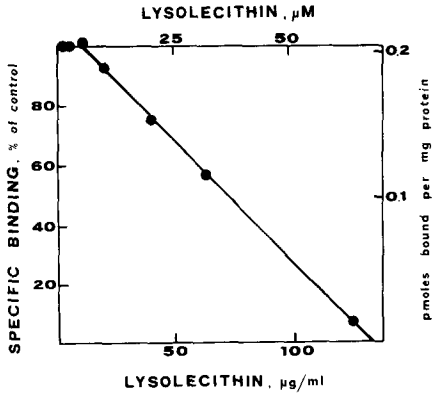

Fig. 2. Inactivation of specific opiate binding by treatment of rat brain membranes with L-α-lysophosphatidylcholine (lysolecithin). A suspension of membranes (0.79 mg protein/ml) was incubated at 37°C for 10 min with different concentrations of lysophosphatidylcholine, then with [^{3}H]diprenorphine (0.85 nM). Specific binding was measured by filtration assay.

The effect of melittin on subsequent opiate binding was tested in separate experiments. Melittin (5–100 μg/ml) used alone in the absence of $CaCl_2$ was without effect *.

2. Solubilization of [^{3}H]opiate-receptor complex with detergents

Solubilized [^{3}H]opiate-receptor complex was measured with a gel filtration assay which permits adequate separation of bound and free ligand. Fig. 3 illustrates the result obtained when the supernatant of a Brij 36 T solubilized preparation was passed through such a column. The bound

* Due to the very small (0.1–0.2%) contamination of even our purest preparation of melittin with phospholipase activity, this effect could not be studied in the presence of calcium chloride.

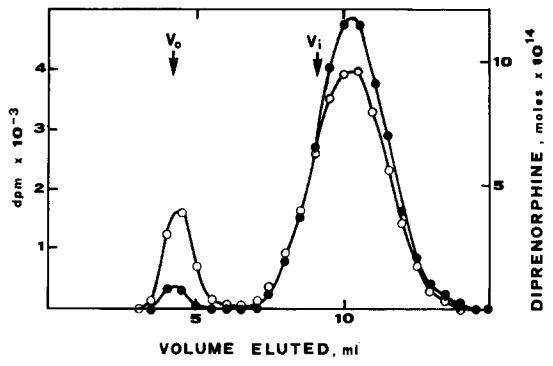

Fig. 3. Assay for solubilized opiate-receptor complex. Samples (0.9 ml) of the 30000×*g* supernatant of solubilized ^{3}H-labelled complex were subjected to gel filtration at 4°C on columns of Sephadex G-50 equilibrated with buffer A. The elution profiles shown (0.5 ml fractions) were obtained with a [^{3}H]diprenorphine complex solubilized with Brij 36 T (2 mg/ml). Total binding (○——○) and non-specific binding (●——●, in the presence of 10^{-6} M levorphanol). Specific binding corresponds to the area between the two peaks at V_0. The columns were calibrated with Blue dextran (V_0 marker) and $Na_2{}^{35}SO_4$ (V_i marker).

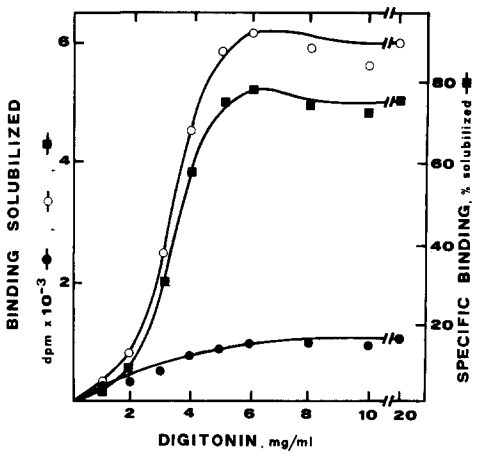

Fig. 4. Solubilization of [^{3}H]opiate receptor complex from rat brain membranes with digitonin. Membranes (protein concentration: 1.17 mg/ml) were prelabelled with [^{3}H]diprenorphine (1.87 nM) and solubilized at 0°C for 30 min with varying concentrations of digitonin. After centrifugation at 30000×*g*, the supernatants were assayed for bound and free opiate. Total binding (○——○) non-specific binding (●——●); specific binding (■——■). Percent solubilization refers to the amount of membrane bound receptor labelled in parallel and tested by filtration assay.

opiate eluted at the void volume of the column while the free opiate was retarded, eluting after sulfate ion used as a marker of the internal volume. The extent of the non-specific binding determined in the presence of levorphanol (1 μM) was in the order of 15–20% of the total binding.

Several detergents were effective in solubilizing [^{3}H]diprenorphine-receptor complex (Table I). In most cases, some of the ligand-receptor complex remained membrane-associated. Due to dissociation, recovery of complex never reached hundred percent. Most detergents solubilized about equal amounts of protein and ligand-receptor complex. However, Zwittergent and octyl-β-D-glucoside extracted over 70% protein. On the other hand, these two detergents were inefficient in solubilizing much of the complex (Table I). The powerful detergent sodium dodecyl sulfate showed even more extreme properties: it solubilized the entire membrane but no stereospecifically bound ligand could be detected in the supernatant.

All other detergents were also tested at 3–5-fold higher concentrations then the ones listed. They did not solubilize more complex nor protein. A plateau of solubilized complex and protein was reached at the concentrations given in Table I. As an example, this is shown for the most efficient detergent, digitonin, in Fig. 4. As can be seen, no more complex is solubilized at digitonin concentrations above approx. 5 mg/ml. When [^{3}H]etorphine was used to prelabel the membrane bound receptor, very similar results were obtained.

Solubilized preparations were usually centrifuged at 30000×*g*. When the centrifugation was carried out at 100000×*g*, no reduction of [^{3}H]opiate complex was observed. In addition, gel filtration of 30000×*g* supernatants of Brij 36 T or digitonin solubilized material on Sepharose 6B columns (1.5×143 cm) gave in both cases a symmetrical peak of specific binding eluting at M_r of about 450000–500000 (not shown).

3. Solubilization of [^{3}H]opiate-receptor complex with bee venom components

When rat brain membranes were first treated with phospholipase A_2 (0.1–20 units/ml) and the 30000×*g* supernatant was subjected after incubation with [^{3}H]etorphine to the gel filtration assay, no stereospecific binding could be detected. When the membranes were first labelled with opiate and then subjected to bee venom phospholipase treat-

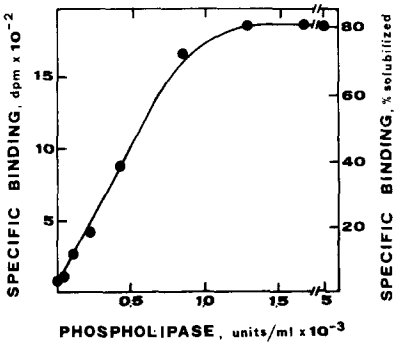

Fig. 5. Effect of bee venom phospholipase on solubilization of the [^{3}H]opiate receptor complex from rat brain membranes. Membranes were labelled with [^{3}H]etorphine (0.77 nM) in the presence or absence of 10^{-6} M levorphanol and treated for 30 min at 0°C with phospholipase A_2 in the presence of 5 mM $CaCl_2$ and melittin (0.3 mg/ml). After addition of EDTA (final concentrations 20 mM) and centrifugation (30000×g), the supernatants were assayed for bound and free ligand. Percent solubilization refers to membrane bound receptor.

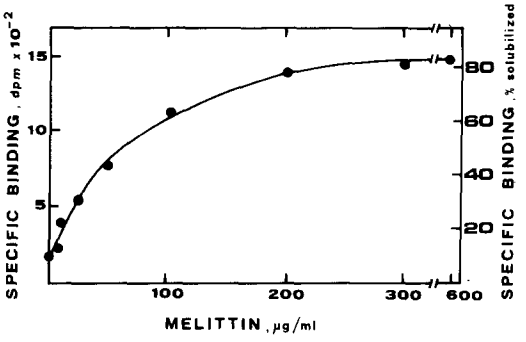

Fig. 6. Effect of melittin on solubilization of the opiate receptor complex from rat brain membranes. Conditions were as in the legend to Fig. 5 but phospholipase concentration was kept constant (1360 units/ml). Percent solubilization refers to membrane bound receptor.

ment (1 to 2000 units/ml) in the presence of 5 mM $CaCl_2$, a maximum of 3% of specifically bound ligand was detected in the supernatant. By comparison, total bee venom (6 mg/ml; containing approx. 1100 units/ml of phospholipase A_2) gave a higher yield of solubilized [^{3}H]opiate-receptor complex (26–32%) but no single venom constituent (hyaluronidase, melittin, apamin) used alone (1–6 mg/ml) was effective in this respect.

The combination of bee venom phospholipase A_2 and melittin did solubilize the prelabelled receptor in high yield. Figs. 5 and 6 illustrate this effect on a [^{3}H]etorphine-receptor complex. Increasing solubilization was observed in the presence of melittin (0.3 mg/ml) when the phospholipase concentration was increased (Fig. 5). At about 1200 units/ml (0.5 mg/ml) a plateau was reached corresponding to solubilization of about 80% of the complex. The effect of different melittin concentrations in the presence of a constant concentration (0.5 mg/ml) of phospholipase is illustrated in Fig. 6. The maximal effect was reached with 0.3 mg/ml of melittin. When the same experiments were performed in the presence of EDTA (10 mM), less than 2% solubilization of ligand-receptor complex was obtained at optimal concentrations of both enzyme and melittin.

Ligand-receptor complexes solubilized by synergistic action of melittin and phospholipase behave as truly solubilized species. They do not sediment at 100000×g and elute from a Sepharose 6B column as a symmetrical peak at M_r 490000 (not shown).

As lysophosphatides generated by phospholipase action presumably act as detergents, solubilization of complex was also studied by lysophosphatidylcholine. For a membrane concentration of 1.2 mg protein per ml, a maximal solubilization of 70% of the complex was obtained with 8 mg/ml of lysophosphatidylcholine (Table I), a result comparable to the one obtained with digitonin (Fig. 4). Lysophosphatidylcholine, however, gave rise to high non-specific binding (50–60% of total binding).

Discussion

Membrane bound hormone or neurotransmitter receptors, such as β-adrenergic [20], nicotinic cholinergic [21] and gonadotropin receptors [22,23], show a reduced affinity for their ligand after phospholipase A_2 treatment *. According to these reports, phospholipids are not directly involved in ligand binding. They rather appear to interact with the receptor in such a way as to modulate its functional activity. In the case of the opiate recep-

* On the other hand, the number of insulin binding sites of cells or membranes increases after treatment with phospholipases [24] possibly because of 'hidden' receptors.

tor, lipids might play a somewhat more active role [10].

As has been shown earlier, opiate binding activity of rat brain membrane preparations is very sensitive to phospholipase A_2 from *Vipera russelli* [5,6,8], as well as from bee venom [7,8], but quite insensitive to phospholipase A_2 from *Crotalus adamanteus* [6,8].

We report here that four phospholipases of A_2 specificity but of different origin lead to complete loss of high affinity opiate binding to brain membranes. This inactivation occurs at similar enzymatic activities (Fig. 1), suggesting that the inactivation is due to phospholipase action and not to activities of undetected minor enzymic contaminants. Additional evidence in favor of phospholipid hydrolysis comes from the finding that all four enzymes tested were inactive in the absence of calcium chloride but were stable to heat. These two properties, absolute requirement of calcium ion and heat stability, are well documented for most types of phospholipase A_2 (Refs. 25, 26 and references therein).

The end products of phospholipid hydrolysis, the fatty acids and lysophosphatides, have been shown to remain membrane associated after phospholipase A_2 treatment [23]. Therefore, these compounds might well be responsible for the observed inhibition of opiate binding. This interpretation is favored by the observation that albumin, known to bind to these compounds with high affinity [23,27], can be used to reverse the inhibition caused by the action of phospholipase A_2 [6,8]. This is confirmed by our finding that higher enzymatic activities are needed for opiate receptor inhibition when albumin is used (Fig. 1B). However, complete protection cannot be achieved, even with albumin concentrations as high as 20 mg/ml. We conclude from these observations that an extensive removal of phospholipids causes irreversible membrane alterations, incompatible with the maintenance of opiate receptor binding.

As expected, addition of end products of phospholipid hydrolysis to the membranes causes inhibition of binding. In the case of fatty acids, high concentrations (1–10 mM emulsions) are required to cause inhibition (Lin, H.K. and Simon, E.J., unpublished data). As for lysophosphatidylcholine, inactivation of the receptor occurs already at 10 μM and complete inhibition is reached at 70 μM (Fig. 2). A similar effect of lysophosphatides has been observed with the gonadotropin receptor [23], although inhibition never reached completeness. The inactivation observed is not due to opiate receptor solubilization since at least 500 μM lysophosphatidylcholine is necessary for this purpose (Table I). The fact that fatty acids and in particular, lysophosphatides, are inhibitory to opiate binding, suggests that inactivation is due to the products formed by phospholipase A_2.

A convenient but slow gel filtration assay was used for the separation of bound and free ligand (Fig. 3). With this assay, we found that several detergents solubilized an opiate-receptor complex with efficiencies similar to the ones reported earlier for Brij 36 T [9]. Digitonin was most efficient in this respect (Fig. 4). Optimal detergents seemed to solubilize about the same amount of ligand-receptor complex and membrane protein and higher detergent concentrations did not extract more protein or prelabelled receptor. Very similar effects have been observed with sodium cholate [12]. Detergents with a more pronounced effect on protein solubilization did not permit recovery of any ligand-receptor complex (octyl-β-D-glucoside, Zwittergent, Table I). It seems therefore that an optimal detergent for solubilization of the opiate receptor has to act in a mild way without denaturating the membrane proteins too much.

Total bee venom solubilizes about 30% of an opiate-receptor complex whereas none of the individual purified bee venom constituents tested were effective in this respect. This indicates that some of the components of bee venom might act synergistically. Phospholipase A_2 and melittin are known to interact in such a way [13,28–32]. The synergistic effect of melittin presumably consists in making the substrate more readily accessible to the enzyme [13]. We have shown that with optimal proportions of these two components, a maximum of 80% of ligand-receptor complex could be extracted from the membranes prelabelled with [^{3}H]opiate (Figs. 5 and 6). This corresponds to a 30–40-fold increase in solubilization when compared to the action by the same amount of phospholipase alone. Lysophosphatidylcholine, one of the products of phospholipid hydrolysis, solubilizes a similar amount of ligand-receptor complex

(Table I), suggesting that it is this compound formed in situ during the concerted action of phospholipase and melittin that is responsible for solubilization.

In no case were the above-mentioned agents of any use to solubilize free receptors as active entieties still able to bind ligands in solution. Using Triton X-100 as solubilizing agent and removal of excess detergent by adsorption to a hydrophobic matrix, Bidlack et al. [33,34] have recently described solubilization and purification of an opiate receptor from rat brain. With a variety of very similar procedures, we have not been able to detect significant amounts of stereospecific binding (Hiller, J.M. and Simon, E.J., unpublished data). A stable unligated receptor was solubilized from neuroblastoma × glioma hybrid cells as well as from bovine brain with a new synthetic amphoteric detergent [35]. We have recently shown that opiate receptors from a non-mammal, the toad, can be solubilized in active form with digitonin [36,37].

Acknowledgements

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