Biochimica et Biophysica Acta, 1110 (1992) 65–74

BBAMEM 75744

Enrichment of saturated fatty acid containing phospholipids in sheep brain serotonin receptor preparations: use of microwave irradiation for rapid transesterification of phospholipids

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(Received 1 May 1992)

Key words: Serotonin receptor; Phospholipid; Saturated fatty acid; Transesterification; Microwave irradiation; (Sheep brain)

During enrichment of the 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT)-binding serotonin 5-HT_{1A} receptors from sheep brain gray matter (membrane isolation, detergent solubilization and reconstitution into vesicles) a consistent and striking increase in the composition of saturated fatty acids was observed in phospholipids which were coisolated with the receptors. A rapid procedure has been developed for the methylation of free and phospholipid linked fatty acids which were thus analyzed by gas chromatography-mass spectrometry (GC/MS). Esterification of free fatty acids and transesterification of phospholipid linked fatty acids were achieved with 14% boron trifluoride in methanol (BF_3-CH_3OH) in 20 s and 50 s, respectively, under low power microwave irradiation (60 W) with a post-reaction cooling of < 5 min. This is in contrast to the conventional method of heating in a boiling water bath for 10–15 min with BF_3-CH_3OH which is inevitably preceded by time-consuming and inconvenient clamping of vials and followed by cooling for 10 min before the vials can be safely opened. Analysis of fatty acid profiles in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) from egg yolk, phosphatidylinositol (PI) from bovine liver and phosphatidylserine (PS) from bovine brain by both techniques showed comparable results. During detergent solubilization of sheep brain gray matter, the overall proportion of saturated fatty acids in PE (major lipid), PI, PC (major lipid) and PS increased from 50–60% in sheep brain phospholipids to 70–75% in 1.5% CHAPS solubilized, reconstituted and biologically active serotonin 5-HT_{1A} preparations. In sharp contrast, the proportions of saturated fatty acids in 1.5% Triton X-100 solubilized PE (48.1%) (major lipid), PI (63.6%), PC (60.6%) (major lipid) and PS (62.2%) were not significantly different from those in the original sheep brain membranes. Strikingly, this was coupled with the occurrence of very low levels of 5-HT_{1A} receptor activity in the Triton X-100 solubilized preparations. The abundance of 5-HT_{1A} sites in the enriched vesicles obtained only from the CHAPS-solubilized preparations was further confirmed by specific radiolabeling of a 58-kDa polypeptide by the 5-HT_{1A} specific ligand *p*-aminophenylethyl-*m*-trifluoromethylphenylpiparazine (PAPP) which was coupled to a ^{125}I-labeled, photoreactive, heterobifunctional cross-linker, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD). Thus CHAPS-solubilized 5-HT_{1A} receptor preparations are depleted in the more rigid lipids such as sphingolipids and cholesterol, (Banerjee et al. (1990) Biochim. Biophys. Acta 1044, 305–314), but are enriched in vesicle-stabilizing, phospholipid-linked saturated fatty acids which in turn probably stabilize the heptahelical, membrane bound 5-HT_{1A} receptor.

Introduction

Lipids are major constituents of all organisms. While neutral lipids, mainly triglycerides are involved in energy storage, phospholipids form the fabric structure of a cell membrane. The molecular structure (phospholipid head groups) as well as association of different fatty acids in the glycerol backbone of phospholipids determine the physiochemical properties of membranes including packing density, molecular shape and cross sectional characteristics, solid-state behavior and thermotropic phase transitions [1,2]. Therefore, structural characterization of phospholipids is essential to understand the interactions of phospholipids with cholesterol, proteins and metal ions, the processes which are crucial in maintaining the integrity and function of biological membranes [3,4].

We have previously shown that 5-HT_{1A} receptor enrichment leads to the enrichment of PC, PE, PS, PI

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; Tr X-100, Triton X-100; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate; PAPP, *p*-aminophenylethyl-*m*-trifluoromethylphenylpiparazine; PEI, polyethylenimine; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

and PA at the expense of sphingolipids and cholesterol [5] and now we test the hypothesis that the composition of phospholipids in the vicinity of G-protein linked receptors could differ from that in lipids in other membrane domains. Coenrichment of specific phospholipid(s) has been reported during the solubilization of proteins like glycophorin [6–8], the erythrocyte anion channel [9], and Na^+/K^+-ATPase [10]. It has also been demonstrated that intrinsic membrane proteins are released from erythrocyte ghosts along with a mixture of phospholipids, the composition of which differs from that of total red cell lipid [11–13]. These findings could signify interactions in the intact membrane between the extracted proteins and lipids and/or between the nonextracted proteins and lipids, which would survive exposure to detergents. Alternatively, a cosolubilization of specific lipids could reflect a similar affinity of a detergent for those proteins and lipids which would enter the same micelle independently of each other. This subject has been addressed in a systematic study [14] which shows that the presence of membrane proteins in red cell ghosts (as compared to liposomes formed only from red cell lipids) increase solubilization of all membrane phospholipids and cholesterol by Tr X-100, thus reflecting an augmenting effect of at least certain membrane proteins on cosolubilization membrane lipids by detergents. However, it is not certain if such an effect would be observed during membrane solubilization by a detergent which is completely different from Tr X-100.

Therefore, a comparative study of Tr X-100 with the ability of a structurally and otherwise (cmc, charge, hydrophobicity, etc.) different detergent such as CHAPS to solubilize membrane lipids along with membrane proteins is essential. The seven-transmembrane-helix-containing integral membrane protein, serotonin 5-HT_{1A} receptor, has been used as a marker, and our analysis of both total lipid profile and fatty acid compositions of the constitutent phospholipids by GC/MS clearly shows a specific trend in membrane protein and lipid cosolubilization by the two detergents tested.

In order to undertake this task of analyzing a large number of phopholipid bands, we had to devise a rapid and efficient transesterification method using the traditional 14% boron trifluoride in methanol [15] and taking advantage of the fact that the commercially available domestic microwave oven can be used to accelerate the rate of organic reactions. The high efficiency of microwave irradiation results in a dramatic reduction in reaction time [16] and this has been used for the rapid hydrolysis of bile acid methyl esters, peptides and proteins [17,18]. Microwave technique has also be used to reduce the reaction times for Diels-Alder, Claisen and Ene reactions [19] as well as hydrolysis of benzamide, oxidation of toluene, esterification of benzoic acid with methanol and the SN_2 reaction of 4-cyanophenoxide with benzyl chloride in methanol [20]. We now report that it can be used for the transesterification of lipids and we have used the method to study fatty acid profiles of phospholipids in detergent solubilized preparations.

Materials and Methods

Materials. SASD and CHAPS were obtained from Pierce (Rockford, IL); $Na^{125}I$ was procured from ICN and chloramine T was purchased from Sigma; PAPP, serotonin and 8-OH-DPAT were obtained from Research Biochemicals. Palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, arachidonic acid and their corresponding methyl esters were purchased from Sigma (St. Louis, MO). A solution of 14% boron trifluoride in methanol and egg yolk phosphatidylcholine were also obtained from Sigma. Phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were procured from Avanti Polar-Lipids (Alabaster, AL). The microwave oven used for this study has a total capacity of 600 watt (Model M84TMA, Amana, IA). Gas chromatography/mass spectrometric analysis (GC/MS) were done on a Model 5890 Gas Chromatograph coupled to a Model 5970 Mass Selective Detector (Hewlett Packard, Palo Alto, CA). The capillary column used for the analysis of fatty acid methyl esters was fused silica crosslinked with 5% phenylmethyl silicone with a 0.33 nm film thickness.

Transesterification and GC/MS analysis. Esterification of fatty acids or transesterification of phospholipids were carried out in reactivials with a total capacity of 5 ml and cone capacity of 0.9 ml (Pierce, Rockford, IL). We added 0.2–0.5 mg of fatty acid or phospholipids to 2 ml of boron trifluoride in methanol in the reaction vials. For the analysis of fatty acid composition of phospholipids in serotonin receptor preparations, the purified phospholipid band (adsorbed in the silica gel) of TLC plates was scraped off and directly added to the reagent for transesterification. The reaction vials were capped under nitrogen with mini inert valves. For microwave irradiation, we used the lowest power setting of the oven (60 W) and the time periods mentioned in the text. While following the conventional technique, the vials were heated in a boiling water bath for 15 min and then allowed to equilibrate at room temperature (23°C) for 20 min. Fatty acid methyl esters (FAME) prepared from individual fatty acids were deliberately extracted in ether instead of hexane in order to detect unreacted fatty acid. However, FAME prepared from fatty acids showed only one spot on TLC, indicating the quantitative conversion. Therefore, they were analyzed by GC/MS without further purification. FAME prepared from various phospholipids were extracted in hexane and purified using silica gel columns which were eluted with hex-

ane/diethyl ether (80:20, by vol). For GC/MS analysis of FAME, the initial oven temperature of the Gas Chromatograph was maintained at 130°C, and after 1 min of injection, the temperature was raised at a rate of 2 C°/min to 200°C, following which the rate of heating was increased to 9 C°/min to reach a final oven temperature of 280°C.

Buffers. Buffer A: 50 mM Tris-HCl (pH 7.4), 0.32 M sucrose; Buffer B: 50 mM Tris-HCl (pH 7.4) containing 1 mM EGTA, 5 mM $MnCl_2$, 5 mM ascorbic acid and 10 μM pargyline; Buffer C: 25% ethylene glycol in Buffer B; Buffer B_a, 20 mM Tris-HCl (pH 7.4), 0.4 mM EGTA, 2 mM $MnCl_2$, 2 mM ascorbic acid and 0.4 μM pargyline.

Solubilization and reconstitution of sheep brain serotonin 5-HT$_{1A}$ receptor. Enrichment of the 5-HT_{1A} sites in sheep brain gray matter, detergent solubilization of membranes and chloroform/methanol extraction of lipids were carried out as described earlier [5]. Briefly, sheep brain gray matter from freshly slaughtered animals was polytron-homogenized in ten volumes of 50 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose at 4°C (this is the homogenate, SBH), the suspension centrifuged at $1000 \times g$ for 10 min and the pellet obtained was washed three times with deionized water, resuspended to a protein concentration of about 8–10 mg/ml in water (this is the enriched pellet, SBP) and stored at −70°C until use. Before detergent solubilization, SBP was centrifuged at $100000 \times g$ for 20 min, the supernatant discarded, the pellet resuspended in cold (4°C) 50 mM Tris-HCl (pH 7.4) containing 1 mM EGTA, 5 mM $MnCl_2$, 5 mM ascorbic acid and 1 μM pargyline (buffer B), and the mixture supplemented with detergent and serotonin to concentrations of 1.5% and 1 μM, respectively. Following gentle stirring at 4°C for 30 min, the mixture was centrifuged at $100000 \times g$, the supernatant freed from detergent using a Bio-Bead SM2 column (2 ml wet Bio-Bead for every 2-ml detergent extract) and dialyzed against buffer B containing 25% ethylene glycol (buffer C). The [^{3}H]8-OH-DPAT-binding, solubilized and reconstituted serotonin 5-HT_{1A} receptor preparation thus prepared (SBDSE) was centrifuged at $200000 \times g$ and the vesicular pellet obtained was either gently resuspended in buffer C (to obtain an enriched receptor preparation, SBDSP) or in some cases, extracted with 10 ml chloroform/methanol (1:1) for 2 h under nitrogen with stirring. The mixture obtained was sonicated, insoluble matter (proteins) removed by centrifugation, and the supernatant evaporated under nitrogen. The residue obtained was dissolved by sonication in 3 ml 2:1 chloroform/methanol, the mixture centrifuged and the supernatant obtained was evaporated under nitrogen to obtain a mixture of total lipid which was dissolved in 2:1 chloroform/methanol to obtain a 10 mg/ml crude lipid solution. The membrane preparations (sheep brain homogenate, SBH, and enriched membranes, SBP) were extracted with 10 volumes of chloroform/methanol (1:1, and processed as described above in order to obtain total lipid mixtures which were identified, separated and estimated by high performance thin layer chromatography (HPTLC) along with the total lipid mixtures obtained from the detergent solubilized preparations.

HPTLC analysis. HPTLC analysis of lipids was conducted by spotting standard amounts of lipids on the same plate along with the SBH-, SBP-, SBDSP-lipids. The plates (10 × 10 cm) were developed up to 5 cm from the origin with ethyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v), dried at room temperature for about 15 min and then developed full length with hexane/diethyl ether/acetic acid (75:21:4). After drying the plates, the bands were viewed by spraying cupric-phosphoric acid charring reagent (10% $CuSO_4$ in 8% H_3PO_4) and heating at 180°C for 10 min [5] following which the lipid bands were quantitated by densitometry as described earlier [5]. For GC/MS analysis, the HPTLC plates were partially exposed to iodine and the respective lipid bands marked, scraped off and collected from the unexposed parts of the plate.

Binding assay of the receptor activity. The 8-OH-DPAT binding activity was assayed by incubation of a receptor preparation (protein ≤ 1 mg) in Buffer B_a at 23°C in the presence of 1.2 nM [^{3}H]8-OH-DPAT for 20 min (total volume 1 ml). Non-specific binding was determined in the presence of 10 μM serotonin. The bound ligand was separated from the free by filtration through 0.3% PEI-soaked GF/B filter strips and Brandel M 24R cell harvester followed by washes with cold (4°C) 10 mM Tris-HCl (pH 7.4), 1 mM EGTA (3 × 6 ml) [5].

Coupling of SASD to PAPP and radiolabeling of the serotonin 5-HT$_{1A}$ receptor. This step and the next were carried out in the dark, under a 25 watt red light in a fume cupboard [21]. Chloramine T (10 μl of a 10 mg/ml solution in DMSO) was added to a reacti-vial containing a mixture of SASD (2.0 μmol) in DMSO (100 μl), 10 μl of $Na^{125}I$ in 0.01 M NaOH and 3 μl of aqueous KI (3 mg/ml). The vial was immediately stoppered and hand shaken, gently, for 2 min and the solution obtained was transferred to a screw-cap test tube containing a serotonin 5-HT_{1A} receptor-specific ligand, PAPP [22] (0.7 mg, 2.0 μmol in 0.5 ml) in 0.1 M borate buffer (pH 8.4). After 30 min, the total mixture was loaded on a sandwich column made of Amberlite IR-45 (-NH_2) (5 ml)-glass wool-cellulose phosphate (H^+) (5 ml) and the column was eluted with deionized water. The fractions (1 ml) corresponding to the first peak of radioactivity (γ) (fractions 4–6), which contained [^{125}I]SASD-PAPP, were pooled and stored frozen in a sealed tube in the dark at −20°C until use. Completion of the reaction and purity of the product

were checked by silica-gel thin-layer chromatography under red light (using 4% methanol in chloroform for development) and autoradiography (overnight) at −70°C for visualization of the spots. Following autoradiography, the plates were stained with iodine to detect non-radioactive compounds. The purified sample of [^{125}I]SASD-PAPP moved as one major spot at $R_f \sim 0.2$. Conversion of PAPP to [^{125}I]SASD-PAPP was almost quantitative when a 1.5-fold molar excess of SASD was used.

Radiolabeling of the serotonin 5-HT_{1A} sites was carried out by incubating SBDSP (200 μl, approx. 0.4 mg protein) for 20 min at 23°C with 50 μl of [^{125}I]SASD-PAPP (corresponds to approx. 80 nmol PAPP and 100000 cpm/min) in Buffer B_a (total volume 0.8 ml) in the absence and presence of 2 μmol (20-fold excess) and 10 μmol (100-fold excess) of 8-OH-DPAT, and then cooled in ice for 10 min. The samples were then irradiated at 366 nm for 10 min at 4°C using a hand held UV lamp (MINERALIGHT) placed at a distance of 8 cm from the samples. A control sample containing no displacer was left covered in the dark. The resulting mixtures were filtered through Bio-Gel P-2 columns (2 ml each) under centrifugation (using a table-top centrifuge) and the effluents were analyzed by SDS-PAGE as follows.

SDS-PAGE analysis. The radiolabeled samples (100 μl of each), the ^{14}C-labeled protein standards and the affinity chromatography fractions were treated with 0.625 M Tris-HCl (pH 6.8), 20 mM DTT, 1% SDS and 10% glycerol for 3 min at 100°C. After 0.1% SDS-PAGE (10%), the dried gels were subjected to autoradiography at −70°C. Silver staining of a gel run to compare protein profiles of the total reconstituted preparation (SBDSE), the enriched vesicular pellet (SBDSP) and the extra-vesicular supernatant (SBDSS), all obtained from CHAPS-treatment of membranes (SBP) was carried out using a Bio-Rad silver stain kit.

Confirmation of vesicle formation by electron microscopy. The vesicles in SBDSE were centrifuged at 200000 × *g* for 20 min to obtain a pellet (SBDSP) which was fixed with 2% glutaraldehyde in 0.12 M Tris-HCl (pH 7.3) and then treated with 1% OsO_4. Following this, the pellet was embedded in epoxy resin, carefully cut and made into thin sections which were treated with uranyl acetate and Reynolds' lead citrate. Electron microscopy was carried at 60 keV using a Philips 201 electron microscope.

Results

Esterification of fatty acids

In order to determine the time of completion of esterification under microwave irradiation a mixture of three fatty acids was subjected to BF_3-CH_3OH treatment by both conventional method and under microwave irradiation for various time periods and the products were analyzed by HPTLC-charring (Fig. 1). These experiments showed that microwave radiation under the lowest power setting was sufficient to drive the esterification of free fatty acids to completion in 20 s, and the yield was quantitative by both conventional and microwave irradiation methods. Coinjection of authentic methyloleate and oleic acid-methylation products obtained by both conventional and microwave irradiation methods produced a single gas chromatograph peak, and also mass spectra of both the products were identical (Fig. 2) thus establishing microwave-accelerated esterification as an effective alternative of the conventional method of heating.

Palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid and arachidonic acid were converted to their corresponding methyl esters using 14% boron trifluoride in methanol under microwave irradiation. After the reaction, an ether extract of the crude product showed a single spot following TLC, corresponding to a fatty acid methyl ester standard, indicating a complete conversion of the acid to its methyl ester under microwave irradiation. The esterified acid was then further analyzed by GC/MS in order to compare its gas chromatographic retention time and mass spectral characteristics with an authentic sample. For each fatty acid methyl ester thus synthesized, the retention time and mass spectral fragmentation pattern was compared with an authentic sample (Table I). Moreover, coinjection of fatty acid methyl ester prepared under microwave irradiation with the corresponding authentic sample produced only one peak in the total ion chromatogram and the same mass spectral fragmentation pattern, clearly indicating that the synthetic prod-

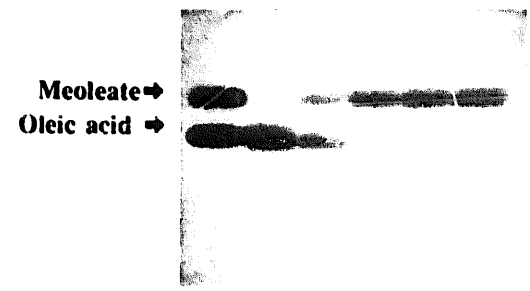

Fig. 1. Time-course of fatty acid esterification by BF_3-CH_3OH treatment under low power microwave irradiation. HPTLC profile of esterification products of free fatty acids: lanes 1 (lipid standards, oleic acid and methyl oleate), 2 (time '0', reaction quenched before microwave irradiation), 3 (2 s), 4 (8 s), 5 (16 s), 6 (20 s). The plate was developed using hexane/ethyl ether/acetic acid (75:24:1, v/v).

uct and the authentic sample have the same chemical identity.

Transesterification of phospholipids

Under low power microwave irradiation, transesterification of PE was complete in 50 s (Fig. 3), and from similar experiments it was also observed that the transesterification profiles of PC, PI and PS were identical to that observed for PE. Transesterification of egg phosphatidylcholine, phosphatidylethanolamine, as well as phosphatidylserine (bovine brain) and phosphatidylinositol obtained from bovine liver, by both microwave

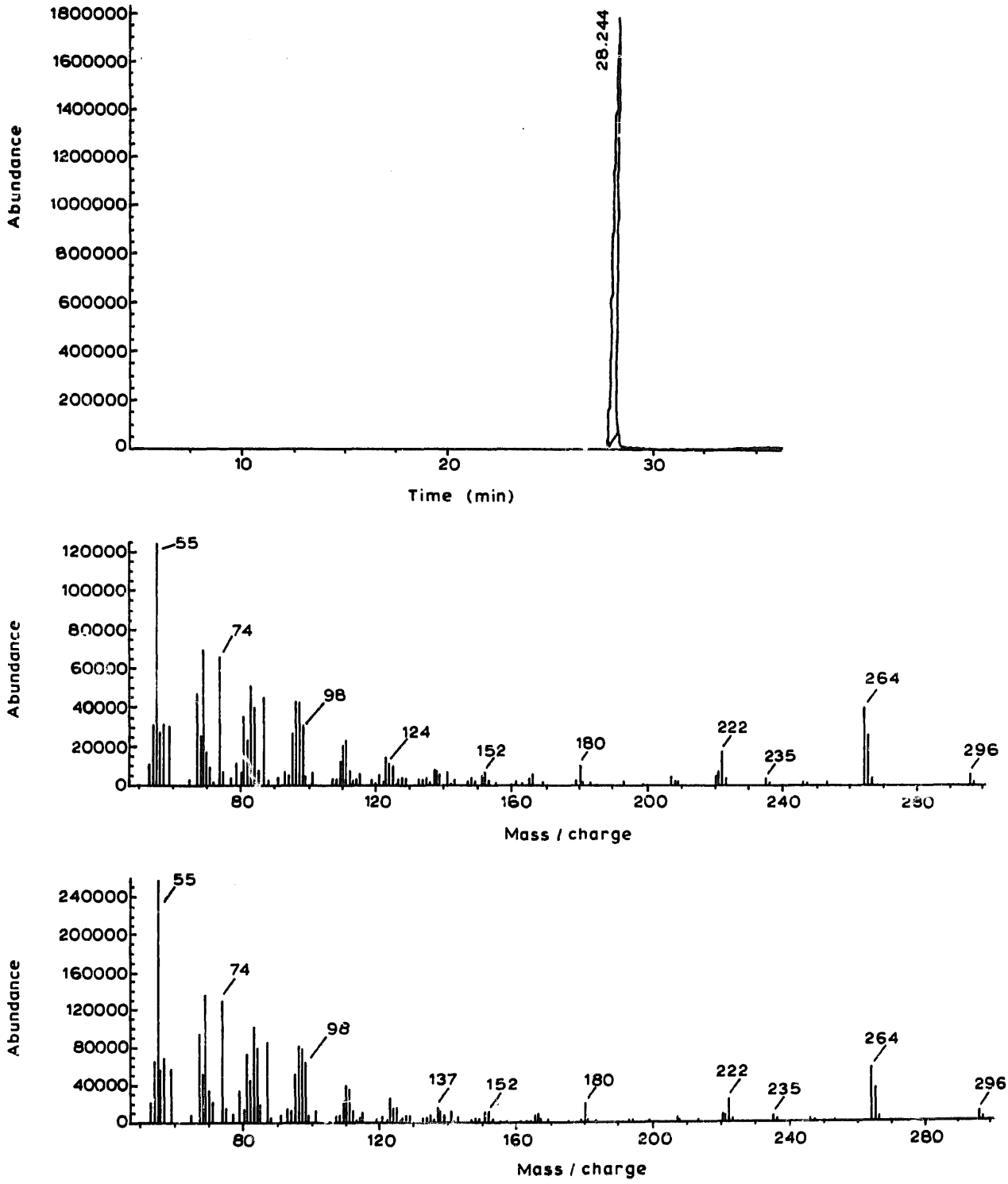

Fig. 2. Methylation of oleic acid by both conventional and microwave irradiation methods yield products with identical mass spectra. (Upper panel) Gas chromatograph peak of authentic methyl oleate coinjected with methylation products of oleic acid obtained by both conventional and microwave irradiation methods. (Middle panel) Mass spectrum of methylation product of oleic acid obtained by conventional method. (Lower panel) Mass spectrum of the methylation product of oleic acid obtained by microwave irradiation method.

TABLE I

Retention times and mass spectral characteristics of authentic samples and fatty acid methyl esters synthesized under microwave irradiation

Fatty acid methyl ester	Retention time		Mass spectral characteristics (m/z)	
	standard	microwave	standard	microwave
Methyl palmitate	20.98	21.00	270 (M^+)	270 (M^+)
			74 (base)	74 (base)
Methyl palmitolate	19.97	19.98	268 (M^+)	268 (M^+)
			74 (base)	74 (base)
Methyl stearate	29.61	29.86	298 (M^+)	298 (M^+)
			74 (base)	74 (base)
Methyl oleate	28.45	28.24	296 (M^+)	296 (M^+)
			55 (base)	55 (base)
Methyl linoleate	28.02	28.03	294 (M^+)	294 (M^+)
			67 (base)	67 (base)
Methyl arachidonate	35.26	35.32	318 (M^+)	318 (M^+)
			79 (base)	79 (base)

technique and conventional technique provided comparable results (Table II). Individual compositions of various fatty acids obtained by both techniques were not statistically different (independent t-test, two-tailed). Interestingly, the total proportion of unsaturated fatty acids often tends to be higher (although not statistically significant) by microwave technique as compared to the conventional method. This might be attributable to little better stability of unsaturated fatty acyl chains during transesterification under microwave irradiation, and thus it offers one more justification for the use of microwave accelerated transesterification.

Phospholipid-linked fatty acids in serotonin receptor membrane and vesicle preparations

The phospholipids obtained from all sheep brain serotonin receptor preparations contained primarily PE (major lipid), PI, PC (major lipid) and PS as determined by HPTLC technique. Fatty acid profiles of individual phospholipids were studied in triplicate by

TABLE II

Fatty acid compositions of various phospholipids

Fatty acid	Percent of total fatty acids [c]							
	egg PE		bovine liver PI		egg PC		bovine brain PS	
	conv [a]	micro [b]	conv [a]	micro [b]	conv [a]	micro [b]	conv [a]	micro [b]
Palmitoleic (16:1)	0.8 (0.2)	0.8 (0.2)	–	–	0.6 (0.2)	0.8 (0.3)	–	–
Palmitic (16:0)	37.6 (3.4)	34.4 (1.5)	42.4 (1.7)	40.9 (1.1)	31.8 (2.5)	31.3 (1.4)	1.8 (0.1)	1.7 (0.3)
Stearic (18:0)	12.4 (0.8)	12.3 (0.8)	19.8 (2.2)	16.5 (1.4)	15.6 (1.3)	14.3 (0.8)	48.2 (0.9)	48.0 (0.6)
Oleic (18:1)	41.8 (3.0)	43.9 (1.7)	32.4 (1.7)	35.9 (1.6)	33.4 (3.9)	32.7 (1.6)	47.9 (0.8)	48.2 (0.3)
Linoleic (18:2)	6.4 (1.1)	7.2 (2.4)	5.6 (1.2)	6.9 (1.1)	13.4 (1.3)	16.0 (2.1)	–	–
Arachidonic (20:4)	1.1 (0.1)	1.4 (0.2)	–	–	5.1 (0.4)	5.0 (0.3)	2.1 (0.1)	2.1 (0.3)
Saturated fatty acids	49.9 (4.2)	46.7 (1.2)	62.3 (3.6)	57.1 (1.5)	47.4 (2.5)	45.7 (1.2)	50.0 (0.9)	49.7 (0.5)
Unsaturated fatty acids	50.1 (4.2)	53.3 (1.2)	37.7 (3.6)	42.9 (1.5)	52.6 (2.5)	54.3 (1.2)	50.0 (0.9)	50.3 (0.5)

[a] Transesterification by conventional technique.

[b] Transesterification under microwave irradiation.

[c] Standard deviation in either case is shown in parentheses, $n = 3$). From independent t-test analysis the difference in results obtained by the two methods was found to be insignificant for each fatty acid.

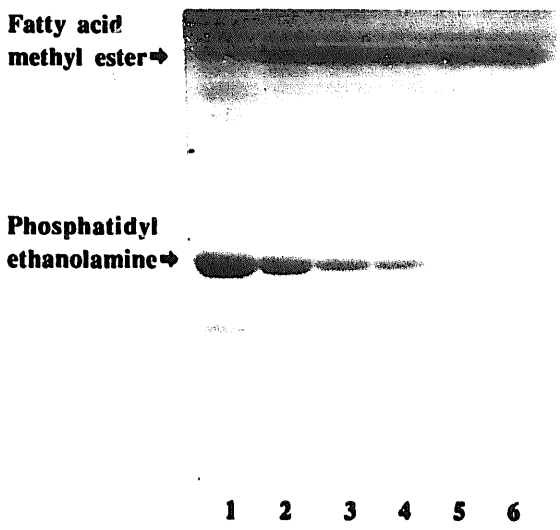

Fig. 3. Time-course of transesterification of phospholipids by BF_3-CH_3O H under low power microwave irradiation. HPTLC profile of transesterification products of PE: lanes 1 (10 s), 2 (20 s), 3 (30 s), 4 (40 s), 5 (50 s), 6 (60 s). The plate was first developed up to R_f 0.8 using ethyl acetate/1-propanol/chloroform/methanol/0.25% KCl (aq): 25:25:20:15:9 dried and then developed full length using hexane/ethyl ether acetic acid (75:21:4, v/v).

TABLE III

Fatty acid composition of phosphatidylethanolamine (PE), Phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylserine (PS) of sheep brain enriched membranes (SBP) containing the serotonin 5-HT_{1A} receptor

Fatty acid q	Percent of total fatty acids [a]			
	PE	PI	PC	PS
Myristic (14:0)	1.4 (0.2)	2.9 (0.4)	5.1 (0.3)	0.5 (0.1)
Pentadecanoic (15:0)	–	1.3 (0.1)	2.7 (0.5)	–
Palmitic (16:0)	20.3 (1.0)	36.6 (3.0)	37.8 (1.0)	36.9 (2.6)
Palmitoleic (16:1)	6.6 (0.3)	10.8 (1.9)	12.0 (1.6)	9.7 (1.1)
Stearic (18:0)	29.1 (1.2)	23.8 (2.3)	11.8 (0.6)	22.7 (1.3)
Oleic (18:1)	35.4 (2.5)	22.4 (1.1)	29.4 (1.2)	20.7 (2.9)
Linoleic (18:2)	2.0 (0.2)	–	–	3.1 (0.2)
Arachidonic acid (20:4)	5.2 (0.4)	2.1 (0.3)	1.1 (0.2)	6.3 (0.5)
Saturated fatty acids	50.8 (2.1)	64.6 (1.4)	57.5 (1.5)	60.1 (1.5)

[a] Standard deviation is shown in parentheses ($n = 3$).

TABLE IV

Fatty acid composition of the detergent-solubilized and reconstituted sheep brain preparations

Fatty acids	Percent of total fatty acids [c]				[^{3}H]8-OH-DPAT binding (cpm/mg protein)
	PE	PI	PC	PS	
(CHAPS)					
14:0	0.8 (0.3)	5.7 (1.4)	0.5 (0.1)	0.4 (0.1)	
15:0	–	3.5 (0.7)	–	–	
16:0	45.1 (2.6)	32.1 (3.4)	50.8 (1.9)	43.1 (1.3)	
16:1	0.5 (0.2)	8.5 (1.2)	0.7 (0.1)	1.4 (0.2)	12551 (±350)
18:0	18.8 (0.9)	32.3 (2.6)	21.2 (0.6)	33.6 (1.8)	
18:1	30.8 (0.9)	17.5 (1.2)	26.8 (1.4)	21.5 (2.1)	
20:1	4.1 (0.4)	0.5 (0.1)	–	–	
Saturated fatty acids [a]	64.7 (2.1)	73.6 (2.2)	72.5 (1.5)	77.1 (2.5)	
(Tr X-100)					
14:0	1.2 (0.3)	0.6 (0.2)	–	–	
16:0	12.0 (1.9)	26.5 (3.3)	40.3 (0.8)	19.0 (1.8)	
16:1	1.3 (0.2)	7.7 (1.0)	1.9 (0.4)	4.1 (1.0)	
18:0	34.9 (0.7)	36.5 (4.1)	20.3 (0.7)	43.2 (1.8)	340 (±17)
18:1	39.8 (0.7)	25.8 (3.1)	35.4 (1.1)	32.1 (1.4)	
18:2	0.7 (0.2)	–	–	–	
20:4	9.9 (1.5)	2.9 (0.5)	2.0 (0.1)	1.6 (0.3)	
Saturated fatty acids [b]	48.1 (2.8)	63.6 (2.6)	60.6 (1.4)	62.2 (2.5)	

[a] Overall proportion of saturated fatty acids in each phospholipid was significantly higher than that in SBP.

[b] Overall proportion of saturated or unsaturated fatty acids in each phospholipid was not significantly different from that in SBP.

[c] Standard deviation ($n = 3$) is shown in parentheses and significance of data was assessed by independent t-test.

[d] Binding assays were carried out in triplicate using 50–100 μl of each solubilized and reconstituted preparation in the presence of 1.15 nM [^{3}H]8-OH-DPAT and $2.75 \cdot 10^5$ cpm/sample [5].

transesterification under microwave irradiation (Table III).

In the enriched sheep brain membranes (SBP), the major fatty acids found in PE, PI, PC and PS were palmitoleic, palmitic, linoleic, oleic and stearic. Arachidonic acid and other polyunsaturated fatty acids were minor components. Only small amounts of pentadecanoic acid were present in PI and PC, and the proportions of fatty acids in the SBP-phospholipids were similar to those of the lipids present in the crude homogenate obtained from sheep brain gray matter (SBH). The overall proportions of phospholipid-linked saturated fatty acids were 50.8% (PE), 64.6% (PI), 57.5% (PC) and 60.1% (PS).

In sharp contrast, after membrane solubilization with CHAPS followed by removal of detergent and reconstitution in the cosolubilized lipids, it was found that the proportions of saturated fatty acids in the CHAPS-solubilized membrane lipids were significantly higher: 64.3%% for PE, 73.6% for PI, 72.5% for PC and 77.1% for PS (Table IV). The CHAPS-solubilized preparations thus obtained also displayed high and consistent [^{3}H]8-OH-DPAT binding activity as reported earlier [5] even after repeated centrifugal separation of the lipid vesicles containing the 5-HT$_{1A}$ sites followed by gentle resuspension. Membrane solubilization in a similar way using Triton X-100 followed by reconstitution, yielded a similar, vesicle-containing preparation. However, the extracted phopholipids contained saturated fatty acids in the proportions of 48.1% for PE, 63.6% for PI, 60.6% for PC, and 62.2% for PS which were not significantly higher than the fatty acid proportions in the SBP-phospholipids. Interestingly, the Triton X-100-solubilized preparations contained virtually no 5-HT$_{1A}$ sites as shown by negligible [^{3}H]8-OH-DPAT binding. For both detergents the solubilization was carried out at an optimum concentration (for the solubilization of both proteins and lipids) of 1.5%, the details of which will be published elsewhere.

The polypeptides which were retained by the biologically active ([^{3}H]8-OH-DPAT binding) vesicles obtained after SBP-solubilization by CHAPS followed by detergent removal, reconstitution by dialysis (unenriched vesicles, SBDSE) and two cycles of centrifugal separation of the vesicles thus obtained (200–2000 Å from electron microscopy, electron micrograph not shown for brevity) at 200000 × *g* (enriched vesicles, SBDSP) were analyzed by reducing 0.1% SDS-PAGE (Fig. 4a, lanes 3 and 4) and compared with the polypeptides in the unenriched vesicles (SBDSE) and the biologically inactive ([^{3}H]8-OH-DPAT binding) first supernatant (SBDSS) obtained after separation of vesicles. The enriched vesicles (SBDSP) displayed a broad band at 58 kDa and two other major polypeptide bands at 40 kDa and 35 kDa, and large amounts of polypeptides which were not retained in the vesicles were found in the first supernatant (SBDSS). Upon radiolabeling of the enriched vesicles (SBDSP) using the heterobifunctional cross-linking agent SASD which was coupled to the serotonin 5-HT$_{1A}$ receptor-specific ligand PAPP [22], only a 58 kDa polypeptide band was found to be radiolabeled. The labeling could be displaced by increasing concentrations of a different serotonin 5-HT$_{1A}$ receptor-specific ligand, 8-OH-DPAT (Fig. 4b). Therefore, the polypeptide band at 58 kDa contains the serotonin 5-HT$_{1A}$ receptor protein as at least one of its components. Since it is unlikely to have a large number of polypeptides with the same molecular weight (58 kDa), it is most probable that a protein complex (receptor + G-proteins + other proteins) formed by the serotonin 5-HT$_{1A}$ receptor and a few other polypeptides (Fig. 4a, lanes 3 and 4) is tightly associated with the lipids in the enriched vesicles (SBDSP).

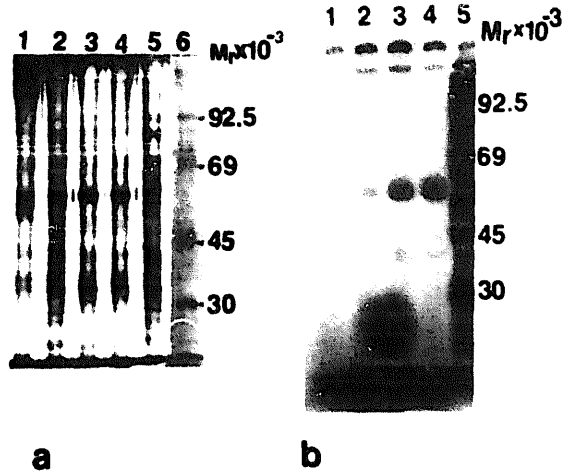

Fig. 4. The serotonin 5-HT$_{1A}$ receptor is firmly associated with vesicles formed from coisolated lipids. (a) 0.1% SDS-PAGE (10% acrylamide) of enriched membranes (SBP) (lane 1, 4 μg protein), CHAPS-solubilized and reconstituted preparation (SBDSE) (lane 2, 12 μg protein), enriched 5-HT$_{1A}$ and lipid preparation (SBDSP) obtained after two rounds of 200000 × *g* separation of vesicles from SBDSE (lanes 3 and 4, 12 μg protein/lane), the first supernatant (SBDSS) obtained from SBDSE after 200000 × *g* separation of vesicles (lane 5, 12 μg protein), and ^{14}C-labeled protein standards (autoradiogram strip obtained from the same gel) (lane 6). Following silver staining major polypeptide bands were observed at 58 kDa, 40 kDa and 35 kDa (a doublet). (b) 0.1% SDS-PAGE (10% acrylamide) of polypeptides radiolabeled by [^{125}I]SASD-PAPP in the enriched vesicles (SBDSP): radiolabeling of the 5-HT$_{1A}$ sites was carried out in the absence (lanes 3 and 4) and the presence of 20-fold (lane 2) and 100-fold (lane 1) excess of 8-OH-DPAT. Molecular weight of the radiolabeled bands were determined with respect to ^{14}C-labeled standard proteins (lane 5). Specific radiolabeling of a 58-kDa polypeptide is observed, and it exactly corresponds in molecular weight to the 58-kDa silver stained band as obtained in (a) after SDS-PAGE of the enriched vesicles (SBDSP).

Discussion

A rapid method for transesterification of fatty acids

GC/MS analysis of fatty acids present in every phospholipid band involved transesterification followed

by extraction of the product and immediate injection (in order to avoid oxidation of polyunsaturated fatty acids) into the GC/MS system. The conventional method of transesterification involves a lengthy process of heating a water bath to 100°C and heating the reacti-vial containing the reaction mixture for 15 min using various clamping arrangements and finally, once the reaction is complete, a wait of 10 min until the contents of the vial come to atmospheric pressure. When the reaction is carried out within 20–50 s in a microwave oven, the contents, which are not heated as much as in the conventional technique, come to atmospheric pressure within 3–5 min, and one can save at least 30 min per sample. The sample can then be extracted, purified and injected into the GC/MS apparatus immediately after the reaction without storage, in order to obtain the most reliable results.

Differential solubilization of phospholipids by Triton X-100 and CHAPS

The total amount of phospholipids extracted from a 2.3-ml aliquot of SBP containing 20 mg protein was 6.3 mg for CHAPS (7 mg of solubilized protein) and only 2.3 mg for Tr X-100 (10 mg of solubilized protein), as determined by densitometry [5]. This was not sufficient to explain the large difference in [^{3}H]8-OH-DPAT binding activity between the solubilized and reconstituted preparations obtained using these two detergents (Table IV). Therefore, it is thought provoking to note that the highly active, CHAPS-solubilized and reconstituted preparation of 5-HT$_{1A}$ sites (SBDSP) also contains strikingly higher proportions of phospholipid-linked saturated fatty acids which might be expected to stabilize membrane and vesicular structures more than the *cis*-double-bonded unsaturated fatty acids. Coupled with this is the fact that only a few major polypeptides along with the serotonin 5-HT$_{1A}$ receptor protein are retained by the enriched vesicles (SBDSP) (Fig. 4a). Implications of this could be that an integral membrane protein such as the serotonin 5-HT$_{1A}$ receptor, which contains seven transmembrane helices, and some other associated proteins (these proteins contain GTP-binding activity and adenylate cyclase activity) (Banerjee et al., unpublished observation) are so strongly associated with the membrane stabilizing, saturated-fatty acid containing phospholipids that those lipids co-solubilize along with the receptor. Thus further separation of the lipid vesicles by centrifugation followed by gentle resuspension would lead to total retention of the serotonin 5-HT$_{1A}$ receptor and the associated proteins. Such co-purification of G-proteins along with adenosine A1 receptors [23] and fatty acids along with the β-adrenergic receptors [24] have been documented before, and enhancement of overall lipid solubilization by detergent-solubilized proteins has also been reported by McDonald [14].

The second alternative could be that CHAPS, because of its ability to preferentially solubilize saturated-fatty acid containing phosphoglycerides which can form tighter and stabler vesicles than the *cis* double bonded unsaturated-fatty acid containing phosphoglycerides, is able to produce such vesicles where solubilized, integral membrane proteins are stabilized and efficiently reconstituted. This, in combination with the superior ability of CHAPS to solubilize the heptahelical, G-protein bound receptors, probably gives rise to the large difference in [^{3}H]8-OH-DPAT binding activity of the CHAPS- and Tr X-100-solubilized and reconstituted preparations.

There is a finite possibility of preferential stabilization of the serotonin 5-HT$_{1A}$ receptor by CHAPS which could be unrelated to the co-solubilization of lipids, since Mestikawy and co-workers reported the solubilization of the serotonin 5-HT$_{1A}$ receptors from rat brain using 0.5% CHAPS without the following step of reconsitution of the solubilized receptor in lipid vesicles [25]. However, in contrast, a soluble form of the bovine brain serotonin 5-HT$_1$ receptor was successfully obtained only after reconsitution in non-endogeneous phospholipid vesicles [26]. In our hands, the sheep brain serotonin 5-HT$_{1A}$ receptor was obtained in a soluble form only when it was solubilized along with endogeneous lipids at 1.5–2% CHAPS and the solubilized [^{3}H]8-OH-DPAT binding activity was three-fold higher when the receptor was preliganded (with 1 μM serotonin or 8-OH-DPAT) before solubilization. A [^{3}H]8-OH-DPAT binding assay of the detergent-extract immediately after solubilization with 0.5% or higher concentrations of CHAPS (the receptor cannot be preliganded in the absence of either post-solubilization dialysis or gel filtration which removes the ligand and frees the receptor for ligand-binding) never produced any discernible activity [5].

Finally, we could never recover any [^{3}H]8-OH-DPAT binding activity by reconstitution in vesicles formed from non-endogeneous lipids (PE/ PC/ PS/ PI/ cholesterol) added in the same proportion as in the active vesicles but to a 1.5% CHAPS solubilized preparation which had been delipidated by Sephacryl-200 gel filtration chromatography. This strongly suggests that there is indeed a lipid-receptor association which could be essential for maintaining biological activity of the sheep brain 5-HT$_{1A}$ receptor. While studies with more detergents are required to reach a firm conclusion, our present report gives a new perspective about CHAPS which has been widely used in recent times for the solubilization of various receptors [5,23,27,28].

Acknowledgements

We thank Mr. J.B. Joo and Mr. Brett Chromy for excellent technical support and Dr. S. Szuchet and Mr.

Paul Polak for generous supplies of sheep brain gray matter. Supported in part by USPHS Grant HD-06426 to G. Dawson.

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