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4′-Amino-benzamido-taurocholic Acid Selectively Solubilizes Glycosyl-phosphatidylinositol-Anchored Membrane Proteins and Improves Lipolytic Cleavage of Their Membrane Anchors by Specific Phospholipases

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Glycosyl-phosphatidylinositol-anchored membrane proteins (GPI-proteins) are normally identified either by cleavage of the lipid anchor using (glycosyl)phosphatidylinositol-specific phospholipases C or D (GPI-PLs) or by metabolic labeling of the lipid moiety with specific building blocks. Therefore, methods for discrimination between transmembrane proteins and GPI-proteins on the basis of their physicochemical properties are desirable. Here we are presenting a selective extraction method for typical well-characterized mammalian GPI-proteins, e.g., acetylcholine esterase, alkaline phosphatase, 5′-nucleotidase, and lipoprotein lipase, using a derivative of taurocholate. The results were compared to those obtained with well-characterized transmembrane proteins, e.g., insulin receptor and hydroxymethyl glutaryl coenzyme A-reductase, glucose transporters, or aminopeptidase M and several commercially available detergents. With regard to total membrane proteins, it was possible to selectively enrich GPI-proteins up to 8- to 14-fold by using concentrations between 0.1 and 0.3% of 4′-NH_2-amino-7β-benzamido-taurocholic acid (BATC). In addition, the cleavage specificity and efficiency of (G)PI-PLs were increased in the presence of identical concentrations of BATC compared to commonly used detergents, e.g., Nonidet P-40. Therefore, the present study shows that the use of BATC facilitates the identification of glycosyl-phosphatidylinositol-anchored membrane proteins.

The number of proteins which are found to be anchored in the plasma membrane of eucaryotic cells by a lipid or glycolipid moiety has been increasing steadily. Among these are structural proteins as well as receptors, surface antigens, cell adhesion molecules, and ectoenzymes (1–5). Some of them are of particular pharmaceutical and biological interest, e.g. variant surface coat glycoprotein from *Trypanosoma brucei* (T.b.)[2] (6–8), T-cell-specific surface antigen from mammalian lymphocytes (9), carcinoembryonic antigen of human tumor cells (10), decay accelerating factor from human blood cells (11), and Scrapie prion protein from hamster brain (12). The membrane anchor of glycosyl-phosphatidylinositol-anchored membrane proteins (GPI-proteins) contain a phosphatidylinositol moiety linked to the carboxy terminus of the polypeptide via nonacetylated glucosamine, three mannosyl residues, and a phosphate ester-linked ethanolamine (13, 14).

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[2] Abbreviations used: GPI-protein(s), glycosyl-phosphatidylinositol-anchored membrane protein(s); (G)PI-PLC(D), (glycosyl-)phosphatidylinositol-specific phospholipase C(D); BATC, 4′-NH_2-7-benzamido-taurocholic acid; NP-40, Nonidet P-40; TX-100, Triton X-100; LPL, lipoprotein lipase; AChE, acetylcholine esterase; aP, alkaline phosphatase; 5′-N, 5′-nucleotidase; GLUT4/1, glucose transporter isoform 4/1; HMG-CoA-R, hydroxymethyl glutaryl coenzyme A-reductase; IR, insulin receptor; ApM, aminopeptidase M; B.c., *Bacillus cereus;* B.t., *Bacillus thuringiensis;* S.a., *Staphylococcus aureus;* T.b., *Trypanosoma brucei;* CRD, cross-reacting determinant; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CMC, critical micellar concentration; CRD, cross-reacting determinant; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Chaps, 3-[C3-cholamidopropyl)dimethylammonio]propanesulfonic acid; AC, adenylate cyclase.

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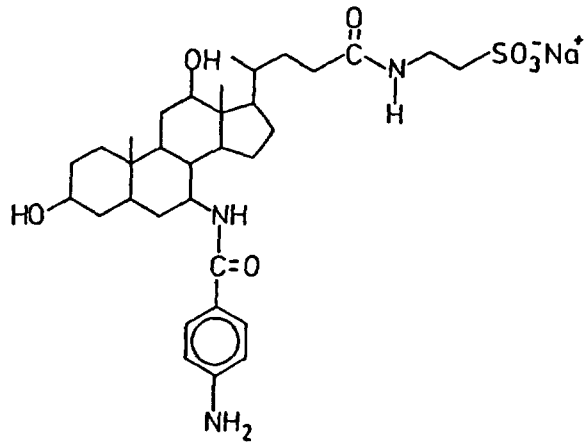

FIG. 1. Molecular structure of 4'-NH_2-7-benzamido-taurocholic acid.

Proteins linked to the core structure are usually identified by the use of the following two methods: (i) cleavage of the GPI-moiety using (glycosyl-)phosphatidylinositol-specific phospholipase C or D [(G)PI-PLC(D)] and monitoring of the release of the protein moiety from the membrane by subsequent TX-114 partitioning, which allows the separation of the amphiphilic GPI-proteins from its released hydrophilic counterpart (15) [successful cleavage by (G)PI-PLC can be monitored by the generation of an inositol-1,2-cyclic phosphate epitope which is recognized by antibodies against this specific determinant (CRD) (16)]; (ii) metabolic radiolabeling of the GPI-protein with characteristic constituents of the core glycan.

By using specific chemical reactions, e.g., nitrous acid deamination and reduction (7), aqueous HF dephosphorylation (17) or hydrolysis with 4 M TFA (18), it is possible to identify characteristic radiolabeled fragments of the core glycan structure. Since these methods are laborious and time consuming, it was desirable to evaluate techniques for discrimination of GPI-proteins from other membrane proteins by specific solubilization. However, the reported enrichment after solubilization with certain detergents (TX-100, TX-114) does not provide enough selectivity for GPI-proteins to allow a clear determination of the type of membrane anchorage (19–22).

It is also known that the type and concentration of the detergent used is critical for lipolytic cleavage of free and protein-linked GPIs by (G)PI-PLs; however, only limited data have been published about the susceptibility of GPI-proteins to GPI-PLs in the presence of detergent and under conditions of differential extraction. This is presumably due to the necessity of substrate presentation within

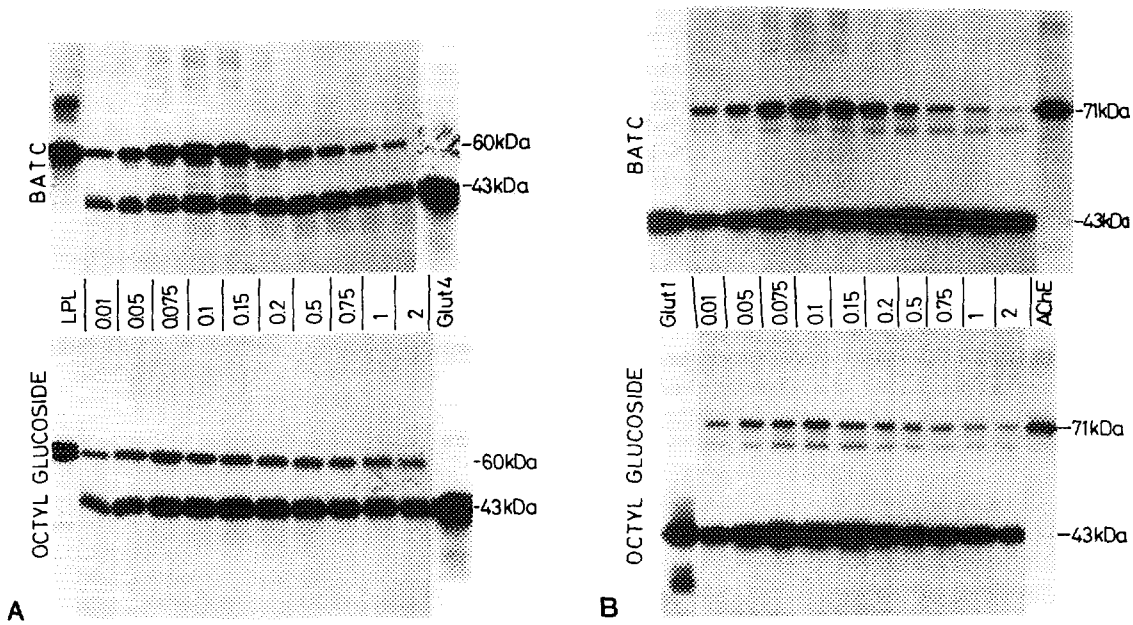

FIG. 2. Differential solubilization of membrane proteins by BATC. (A) Rat adipocytes were metabolically labeled with [^{35}S]methionine. Plasma membranes were prepared and incubated with various concentrations of BATC (upper gel) or octylglucoside (lower gel). After centrifugation, proteins in the supernatants were precipitated with TCA and the acetone-washed pellets were dissolved in immunoprecipitation buffer at identical protein concentration. LPL and GLUT4 were immunoprecipitated simultaneously with anti-LPL and anti-GLUT4 antibodies and protein A–Sepharose. The pellets were analyzed by SDS–PAGE and fluorography. Total LPL and GLUT4 were immunoprecipitated from equivalent amounts of SDS (2%)-solubilized plasma membranes (outer lanes of the gels). (B) Bovine erythrocyte membranes were incubated with various concentrations of BATC or octylglucoside. After centrifugation, proteins in the supernatants were precipitated with TCA. The pellets were dissolved in sample buffer at identical protein concentrations and analyzed by SDS–PAGE, immunoblotting simultaneously using anti-AChE and anti-GLUT1 antisera and ^{125}I-labeled anti-IgG antibodies, and fluorography. Immunoblotted total AChE and GLUT1 from equivalent amounts of SDS-solubilized erythrocyte membranes are shown on the outer lanes of the gels.

TABLE I

Enrichment of Several Membrane Proteins by Solubilization with Selected Detergents

% Detergent	BATC	OG	DC	TX-100	Chaps	BATC	OG	DC	TX-100	Chaps	BATC	OG	DC	TX-100	Chaps
			IR					GLUT4					AC		
0.05	0.99	0.83	0.92	0.72	0.88	0.51	0.52	0.31	0.51	0.51	0.23	0.12	0.19	0.37	0.30
0.075	1.43	0.92	0.57	0.87	0.92	0.52	0.81	0.47	0.72	0.60	0.31	0.26	0.33	0.66	0.45
0.1	1.91	1.05	0.66	1.31	1.19	0.79	1.24	0.48	0.88	0.72	0.58	0.92	0.52	0.74	0.59
0.2	2.33	1.56	1.02	1.55	1.25	0.86	1.46	0.69	0.91	0.81	0.87	1.25	0.77	0.79	0.65
0.3	2.02	1.44	1.35	1.42	1.19	0.95	1.52	0.73	0.95	0.88	0.92	1.25	0.95	0.82	0.79
0.5	1.13	1.37	1.42	1.22	1.17	0.80	1.67	0.75	1.05	0.95	0.97	1.27	1.10	0.90	0.87
0.75	1.04	1.12	1.27	1.20	1.01	0.96	1.20	1.03	1.12	1.10	1.18	1.11	1.21	1.01	1.05
1.0	0.82	1.05	1.15	1.15	0.95	1.10	1.13	1.27	1.21	1.17	1.14	1.03	1.55	1.10	1.22
2.0	1.12	0.77	1.05	1.10	1.12	1.18	0.96	1.49	1.32	1.29	1.13	0.94	1.37	1.20	1.31
5.0	1.00	0.89	0.85	1.12	1.19	1.00	0.95	1.76	1.34	1.25	1.00	1.08	1.30	1.21	1.44
			AChE					LPL					5'-N		
0.05	3.12	1.43	1.75	0.03	0.66	2.54	1.21	0.53	0.02	0.34	1.86	1.02	0.20	0.08	0.80
0.075	3.34	2.56	0.87	0.04	0.71	4.97	1.92	0.81	0.06	0.62	3.50	1.44	0.37	0.13	0.95
0.1	8.97	1.71	1.23	0.12	0.95	10.18	2.17	1.49	0.17	0.89	7.23	1.57	0.72	0.27	1.22
0.2	9.75	1.48	1.37	0.37	1.31	10.84	1.86	1.22	0.55	1.57	12.52	1.76	0.95	0.75	1.60
0.3	10.15	1.35	1.21	0.55	1.34	10.66	1.66	1.17	0.76	1.76	13.17	1.55	1.19	0.88	1.52
0.5	12.46	1.22	1.11	0.61	1.39	10.58	1.51	1.07	0.81	1.98	14.11	1.42	1.23	0.92	1.44
0.75	7.28	1.13	1.05	0.73	1.18	6.17	1.39	1.02	1.07	1.55	8.73	1.24	1.29	0.95	1.39
1.0	3.55	1.34	0.97	0.91	1.07	2.76	1.13	1.09	0.93	1.27	4.42	0.91	1.35	0.91	1.33
2.0	1.64	1.10	0.92	0.88	1.15	1.82	1.02	1.12	0.73	0.92	2.01	0.78	1.44	0.82	1.30
5.0	1.00	1.04	0.98	0.82	1.26	1.00	0.80	1.15	0.77	0.77	1.00	0.71	1.30	0.89	1.30
			aP					ApM					HMG-CoA-R.		
0.05	1.47	1.20	0.44	0.01	0.21	—	—	—	—	—	—	—	—	—	—
0.075	2.12	1.61	0.39	0.02	0.42	—	—	—	—	—	—	—	—	—	—
0.1	4.72	1.33	0.69	0.09	0.66	4.75	5.16	—	4.45	—	6.93	10.22	—	4.92	—
0.2	6.81	1.25	1.17	0.16	0.75	—	—	—	—	—	—	—	—	—	—
0.3	7.66	1.20	1.25	0.29	0.79	—	—	—	—	—	—	—	—	—	—
0.5	7.92	1.16	1.26	0.42	0.89	3.86	3.66	—	3.32	—	3.21	7.77	—	4.30	—
0.75	7.41	0.97	1.41	0.66	1.09	—	—	—	—	—	—	—	—	—	—
1.0	6.01	0.73	1.30	0.73	1.02	1.75	3.34	—	3.27	—	1.82	14.69	—	4.49	—
2.0	3.31	0.62	1.32	0.70	0.93	1.00	3.73	—	3.18	—	1.00	8.77	—	3.54	—
5.0	1.00	0.65	1.27	0.61	0.85	—	—	—	—	—	—	—	—	—	—

Note. Rat adipocyte plasma membranes (LPL, IR, GLUT4, AC), bovine erythrocyte membranes (AChE), rat liver plasma membranes (aP, 5'-N), and rat liver microsomal membranes (HMG-CoA-R) were incubated with various concentrations of detergents and centrifuged. The supernatant was assayed for specific enzymatic activity AChE, LPL, 5'-N, aP, AC, and HMG-CoA-R. The relative amount of IR and GLUT4 per milligram of membrane protein was determined by immunoblotting with anti-IR and anti-GLUT4 antisera, respectively. Relative specific activities or amounts of immunoreactive material were calculated on the basis of the results obtained in the presence of 2% SDS.

detergent micelles. GPI-PLD from mammalian serum accepts exclusively detergent-solubilized GPI-proteins as substrate (11, 23, 24). Similarly, GPI-PLC from T.b. seems to require detergent for its action under most experimental conditions (25). Although bacterial PI-PLCs accept GPI structures as substrate when embedded in intact membranes, their cleavage efficiency is stimulated by low concentrations of TX-100 or sodium deoxycholate. However, at higher concentrations the stimulatory effect is lost and the cleavage reaction may even be inhibited (9, 26). We tried to identify a detergent with higher selectivity for GPI-proteins and better support of lipolytic cleavage compared to commercially available detergents. This goal was achieved with 4'-NH_2-7-benzamido-taurocholic acid (BATC).

MATERIALS AND METHODS

Materials. *myo*-[^{14}C(U)]Inositol (200–250 mCi/mmol), [1-^{14}C]stearic acid (50–60 mCi/mmol), ^{125}I-labeled anti-(rabbit IgG) antibodies from goat (2–10 μCi/μg), Aquasol II, and autoradiographic enhancer ENHANCE (liquid) were bought from New England Nuclear (Dreieich, FRG). *Glycerol tri*[9,10(*n*)-^{3}H]*oleate* (0.6 Ci/mmol), anti-(rabbit IgG) antibodies from goat coupled to agarose, (3-[^{125}I]iodotyrosyl^{A14}) insulin (2000 Ci/mmol) and Hybond C-extra were bought from Amersham-Buchler (Braunschweig, FRG). Partially purified PI-PLC from *Bacillus*

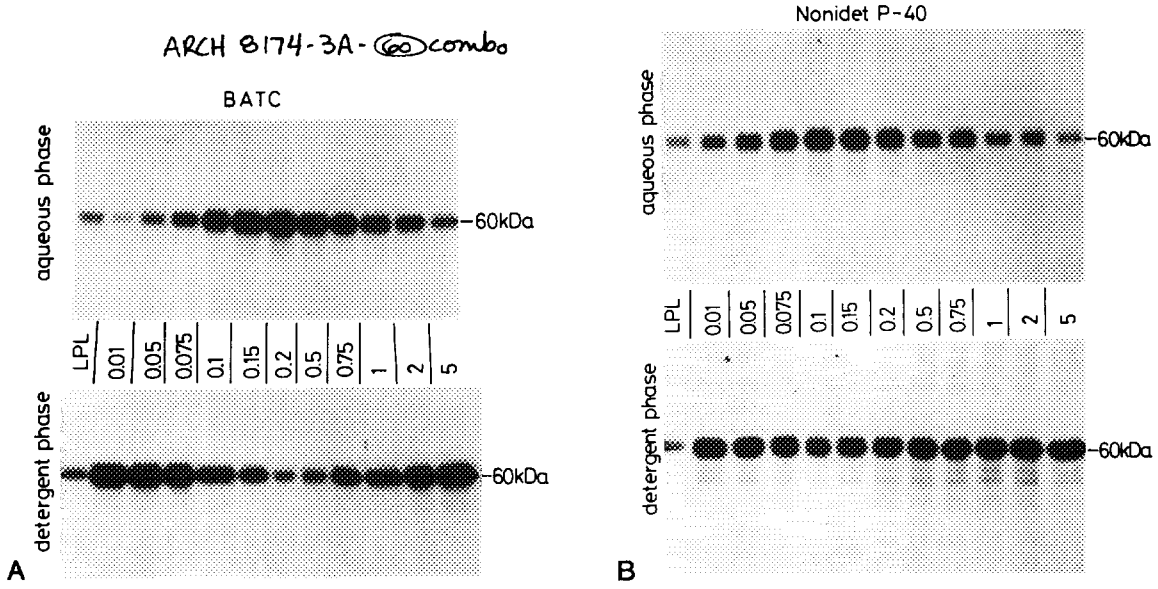

FIG. 3. BATC supports lipolytic cleavage of GPI-protein membrane anchors. Rat adipocytes were metabolically labeled with [^{35}S]methionine and plasma membranes were prepared. The membranes were incubated with 0.1% BATC (A) or NP-40 (B). After centrifugation, the supernatants were diluted 10-fold, adjusted to various concentrations of detergent, and incubated with PI-PLC (B.c.). Samples were subjected to TX-114 partitioning and the separated phases were precipitated with TCA. The pellets were solubilized in sample buffer. The [^{35}S]methionine-labeled samples were immunoprecipitated with anti-LPL antibodies and protein A–Sepharose, followed by analysis of the immunoprecipitates by SDS-PAGE and fluorography. Purified immunoprecipitated LPL was used as a standard (left lane).

cereus (B.c.) and detergents were purchased from Boehringer Mannheim, FRG. Highly purified GPI-PLC (T.b.) and polyclonal anti-CRD antibody against the variant surface glycoprotein from MITat 1.2. were generous gifts from P. Overath (Tübingen, FRG). Crude GPI-PLD from heparinized rabbit serum and pure PI-PLC (B.t. and S.a.) were donated by W. Gutensohn (Munich, FRG). Rabbit antiserum against human milk LPL was kindly provided by Dr. C.-S. Wang (Oklahoma). Collagenase type CLS II was provided by Worthington, (Freehold, NJ). Methionine-free and normal Dulbecco's modified Eagle's medium and reagents for cell culture were bought from Gibco-BRL (Eggenstein-Leopoldshafen, FRG). Affinity-purified anti-GLUT4 and anti-GLUT1 antibodies were directed against a peptide corresponding to the 16 carboxy-terminal amino acids of rat GLUT4 (27) and the 12 carboxy-terminal amino acids of the human GLUT1 sequence (28). Affinity-purified anti-acetylcholine esterase (AChE) antibodies were raised in rabbits against the active hydrophilic papain fragment (68 kDa) of bovine erythrocyte AChE (29, 30), kindly donated by S. Lummers (St. Louis, MO). BATC was prepared at laboratories of Hoechst AG (Frankfurt, FRG) according to published methods (31). All other chemicals were obtained as described previously (32) and of the highest grade commercially available.

Preparation of rat adipocyte plasma membranes. Adipocytes were isolated from epididymal fat pads of 160–180 g male Wistar rats by collagenase digestion under sterile conditions (33) with some modifications (34). The subcellular fractionation was performed according to published procedures (35, 36) with the following modifications: The postnuclear supernatant was centrifuged (12,000*g*, 15 min). The supernatant was centrifuged. The pellet was suspended in 35 ml of buffer A and recentrifuged (1000*g*, 10 min). The washed pellet (12,000*g*, 20 min) was suspended in 5 ml of buffer A, layered onto a 20-ml cushion of 38% (w/v) sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, and centrifuged (110,000*g*, 60 min, 4°C). The membranes at the interface between the two layers (1 ml) were removed by suction, diluted with 3 vol of buffer A and layered on top of an 8-ml cushion of 24% Percoll, 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA. After centrifugation (45,000*g*, 30 min) the plasma membranes were withdrawn with a Pasteur pipet from the lower fourth of the gradient (1 ml), diluted with 10 vol of buffer A, and recentrifuged (150,000*g*, 90 min, 4°C). The pellet was suspended, recentrifuged, finally dissolved in buffer A at 0.5 mg protein/ml, frozen in liquid nitrogen, and stored at −80°C.

Rat liver plasma membranes. These were prepared (37, 38) with the following modifications: The liver removed from one male Wistar rat (250 g) was minced in 150 ml of ice-cold buffer B (25 mM Hepes/KOH, pH 7.4, 250 mM sucrose, 1 mM EDTA) containing antipain, leupeptin, pepstatin (each at 5 μg/ml), PMSF (100 μM), and aprotinin (200 μg/ml), homogenized with 15 strokes in a tight-fitting motor-driven (1500 rpm) Teflon-in-glass homogenizer and then filtered through two layers of nylon mesh and centrifuged (1000*g*, 15 min, 4°C). The washed nuclear pellet was resuspended in 3 vol of buffer B per gram of liver and centrifuged (50,000*g*, 10 min, 4°C). The pellet was suspended in 3 vol of 57% (w/w) sucrose per gram of liver. Twenty-five milliliters of 37.2% (w/w) sucrose was layered onto 25 ml of the 57% sucrose solution. Finally, 8 ml of buffer B was layered above the 37.2% sucrose solution. The discontinuous gradient was centrifuged (120,000*g*, 16 h, 4°C). The plasma membrane fraction appearing at the interface between the 37.2% sucrose layer and the buffer B overlayer was removed, diluted with 4 vol of 10 mM Hepes/KOH (pH 7.5), and centrifuged (150,000*g*, 1 h, 4°C). The membrane pellet was resuspended in buffer B at 1–2 mg protein/mg and stored at −20°C.

Preparation of rat intestinal mucosa homogenate. Total small intestines of five male wistar rats (200–250 g) were taken out and rinsed with ice water and with cold isotonic NaCl solution. Samples were frozen in liquid nitrogen, then, after thawing, the mucosa was scraped off using a spatula. After homogenization in cold 10 mM K,Na-phosphate buffer at pH 7.2, intestinal mucosa samples were kept at −80°C until use.

Preparation of rat liver microsomes. Male wistar rats (200–220 g) were fed a standard diet containing 2% cholestyramine for 10 days under reversed light cycle. The animals were sacrificed on the following day. The minced livers were homogenized in 4 ml/g liver in ice-cold buffer A (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, pH 7.2). The pH was

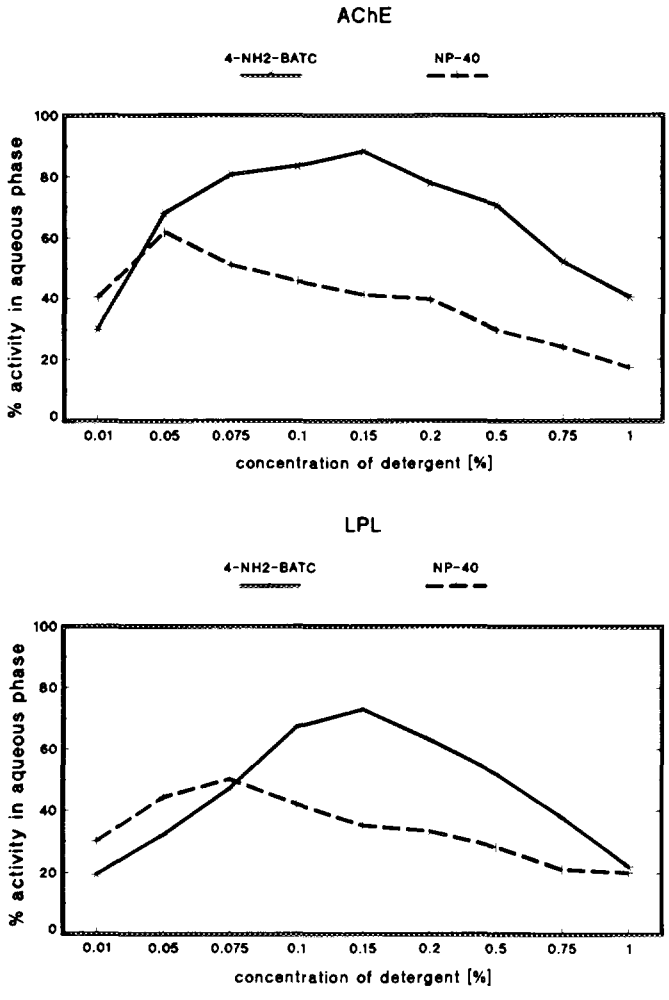

FIG. 4. Concentration dependence of lipolytic cleavage of GPI-proteins in the presence of BATC and NP-40. Rat adipocyte plasma membranes (lower panel) and bovine erythrocyte membranes (upper panel) were incubated with 0.1% NP-40 or BATC and centrifuged. The supernatant was diluted 10-fold, adjusted to various concentrations of the respective detergent, and incubated with PI-PLC (B.c.). The samples were subjected to TX-114 partitioning. The enzymatic activities of AChE and LPL were determined before and after phase separation in the total mixture and aqueous phase, respectively, and are given as percentage total activity recovered in the aqueous phase.

adjusted by the addition of a solution of 0.24 mM EDTA in 1 M KOH. The pellet after centrifugation (12,000*g*, 20 min, 4°C) was washed in the same volume of buffer A. The combined supernatants were subjected to centrifugation (100,000*g*, 60 min, 4°C). The pellet was recentrifuged after homogenization in buffer B (10 mM DTT, 0.1% TX-100, 0.1% BSA in buffer A). The resulting pellet was resuspended in buffer B and was kept frozen at −80°C in 200-μl portions.

Bovine erythrocyte membranes. These were prepared (39) with the following modifications: Washed red cells were suspended in 150 mM NaCl, 5 mM Na-phosphate, 10 mM Tris/HCl (pH 7.5) to an hematocrite of 50%. To 5-ml aliquots 30 ml of 10 mM Tris/HCl (pH 7.5) was transferred and incubated (10 min, 4°C). After centrifugation (50,000*g*, 40 min, 4°C), the membrane pellet was suspended in 30 ml of 10 mM Tris/HCl (pH 7.5) and recentrifuged. This procedure was repeated until the membranes appeared to be colorless (usually four washes). After removal of the last buffer wash, the membranes, suspended in 10 mM Tris/HCl (pH 7.5), were transferred to a new tube. The suspension was adjusted to 5 mg protein/ml with buffer and stored at −20°C.

Solubilization of membrane fractions. Membranes were incubated (30 min, 4°C) with various detergents and concentrations as indicated at a final protein concentration of 0.1 mg/ml under stirring. The samples were layered on top of 0.5 ml of 250 mM sucrose, 8% Percoll, 25 mM Tris/HCl (pH 7.4) per milliliter of solubilized membranes and centrifuged (150,000*g*, 1 h, 4°C). The supernatant above the sucrose layer was carefully transferred to new tubes.

Metabolic labeling of rat adipocytes. Adipocytes were cultured, as described by Marshall and coworkers (40) with modifications. After collagenase digestion, the cells (3×10^7 cells/ml) were washed three times with 4 ml/ml of cell suspension with Dulbecco's modified Eagle's medium (DMEM) containing 1% BSA, 5% fetal bovine serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 1 mg of Fungizone/ml and finally suspended in the same buffer supplemented with 5 mM glucose at 3–5 $\times 10^5$ cells/ml. The radiolabeling was performed by the addition of 250 μCi of *myo*-[^{14}C]inositol or 400 μCi of [^{14}C]stearic acid to 20 ml of cell suspension and incubation (14 h or 1 h, respectively). Subsequently, the cells were concentrated by flotation (1000*g*, 2 min, 25°C), washed three

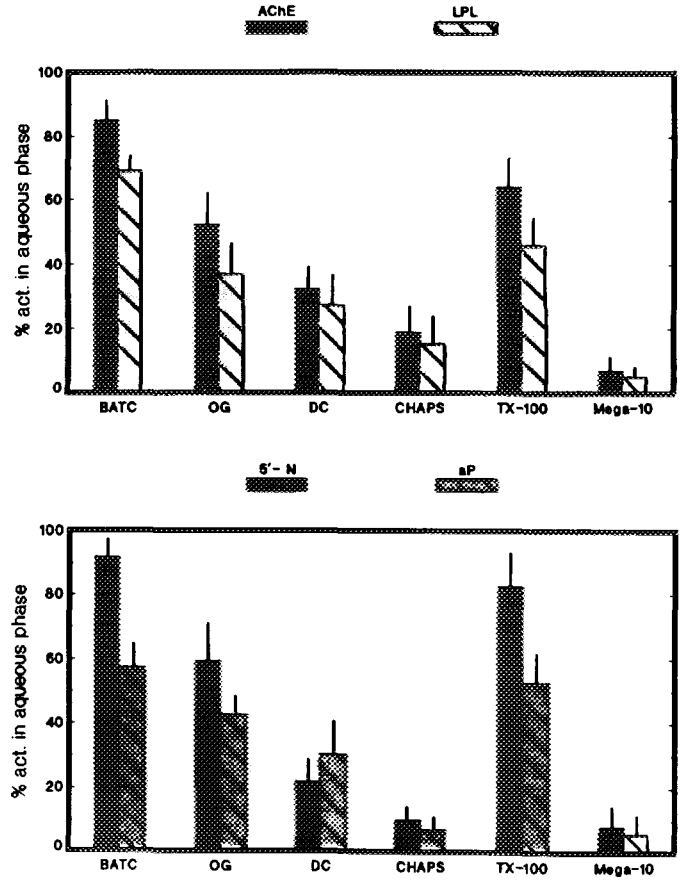

FIG. 5. Influence of selected detergents on lipolytic cleavage. Rat adipocyte plasma membranes (LPL), bovine erythrocyte membranes (AChE), and rat liver plasma membranes (5'-N, aP) were incubated with 1.5% BATC, 0.5% octylglucoside, 2% deoxycholate, 0.5% Chaps, 0.5% TX-100, or 0.75% Mega-10 and then centrifuged. The supernatant was diluted 10-fold and incubated with PI-PLC (B.c.). The samples were subjected to TX-114 partitioning. Enzyme activities were determined and calculated as described above ($n = 4$, ± SD).

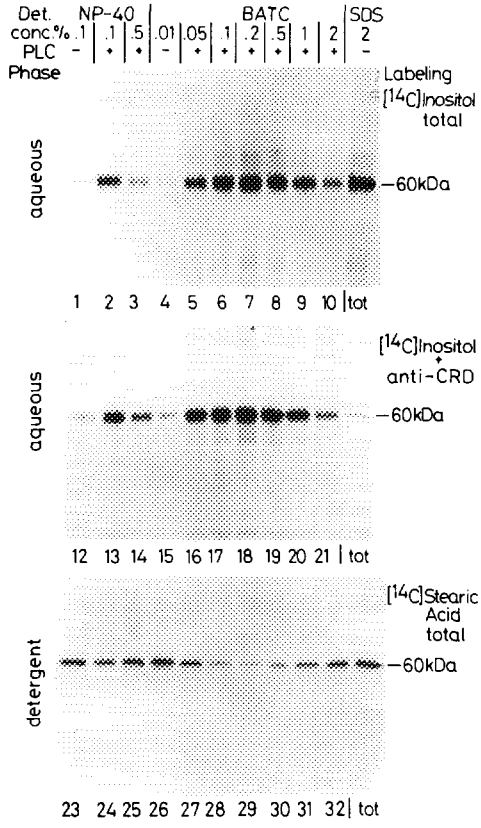

FIG. 6. Cleavage specificity of PI-PLC is not affected by BATC. Rat adipocytes were metabolically labeled with *myo*-[^{14}C]inositol (upper and middle panels) or [^{14}C]stearic acid (lower panel). Plasma membranes were prepared, incubated with 0.1% BATC, 1% NP-40, or 2% SDS, and centrifuged. The supernatant was diluted 10-fold, adjusted to various concentrations of BATC, NP-40, or SDS, respectively, and incubated with PI-PLC (B.c.) as indicated. The samples were subjected to TX-114 partitioning. The aqueous phase of the *myo*-[^{14}C]inositol-labeled samples (lanes 1–10) and the detergent phase of the [^{14}C]stearic acid-labeled samples (lanes 23–32) were immunoprecipitated with anti-LPL antibodies and protein A–Sepharose. The immunoprecipitates were dissolved in sample buffer. The [^{14}C]stearic acid-labeled samples and one-half of the *myo*-[^{14}C]inositol-labeled samples were analyzed directly by SDS–PAGE and fluorography (lanes 1–10 and 23–32). The other half of the *myo*-[^{14}C]inositol-labeled samples was subjected to a second immunoprecipitation using anti-CRD antibodies and agarose-coupled anti-IgG antibodies prior to SDS–PAGE and fluorography (lanes 12–21). As a standard for the total content of *myo*-[^{14}C]inositol- and [^{14}C]stearic acid-labeled LPL in rat adipocyte plasma membranes (tot), equivalent volumes of the total mixtures (before TX-114 partitioning) were used for the immunoprecipitations with anti-LPL (upper and lower panels) and anti-LPL and anti-CRD antisera (middle panel).

times with 20 ml of DMEM, 0.5% BSA, 1 mM *myo*-inositol, or 10 mM stearic acid, respectively, per milliliter of packed cells and finally suspended in buffer A (see above) for preparation of plasma membranes. Labeling with [^{35}S]methionine was performed according to Lawrence and coworkers (41) with modifications: Adipocytes were washed and suspended in methionine-free DMEM containing the same ingredients as indicated above. After addition of [^{35}S]methionine (100 μCi/10 ml of cell suspension), the cells were incubated (1 h, 37°C) then floated by centrifugation (1000*g*, 2 min), washed twice in DMEM containing 10 mM unlabeled methionine, and further processed as described above.

Immunoprecipitation (42). [^{35}S]Methionine-, [^{14}C]inositol-, or [^{14}C]stearic acid-labeled adipocyte plasma membranes were solubilized in sample buffer (60 mM Tris/HCl, pH 6.8, 0.5 mM EDTA, 0.1 mM PMSF, 2% SDS, 2% mercaptoethanol) at 0.5 mg protein/ml, heated (5 min, 95°C), and centrifuged (15,000*g*, 5 min). Fifty microliters of the supernatant was diluted with 1 ml of immunoprecipitation buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% TX-100) and recentrifuged. The supernatant was incubated with 50 μl of anti-rat GLUT4 antibodies or anti-human milk LPL antibodies which had been preloaded onto protein A–Sepharose as described (34). After incubation (1 h, 4°C) under constant shaking, the beads were collected by centrifugation (15,000*g*, 2 min), washed sequentially with 1 ml each of immunoprecipitation buffer containing 1% TX-100/100 mM NaCl, 0.1% TX-100/50 mM NaCl, and 0.1% TX-100. The final pellet with the immunocomplex-containing beads was dissociated in 50 μl of sample buffer (5 min, 95°C) and centrifuged. For a subsequent second immunoprecipitation of the immunoprecipitated LPL with anti-CRD antibodies, 50 μl of the supernatant was diluted with 1 ml of immunoprecipitation buffer. The samples were incubated with 2 μl anti-CRD antiserum (1 h, 4°C) and then with 25 μl of goat anti-(rabbit IgG) antibodies coupled to agarose for 30 min at 4°C under gentle shaking. After centrifugation (15,000*g*, 2 min), the pellet was washed and processed as described above.

Immunoblot analysis. Proteins recovered from the detergent and aqueous phases after TX-114 partitioning were precipitated with 5% TCA and analyzed by SDS–PAGE. Gels were blotted as described previously (43). The blots were blocked with 5% defatted milk powder in phosphate-buffered saline (PBS) for 4 h at room temperature. After addition of anti-GLUT1 (1:200) or anti-AChE (1:500) antiserum, the incubation was continued (16 h, 4°C). The membranes were washed four times (30 min each) with PBS containing 1% TX-100 and then incubated with ^{125}I-labeled anti-(rabbit IgG) antiserum from goat (0.5 μCi/ml) in the same buffer. After four additional washes the blots were dried and subjected to autoradiography.

Digestions with PL's. Membrane proteins (100–250 μg) were incubated in a final volume of 500 μl in the presence of various detergent concentrations for 2 h, 37°C as described (25, 44–46) with the following modifications: 0.5 units of partially purified PI-PLC (B.c.) in 20 mM Tris/HCl (pH 7.2), 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μg/ml BSA; 0.1 units of purified PI-PLC from *Bacillus Thuringiensis* (B.t.) in 10 mM Tris/HCl (pH 7.0), 5 mM potassium phosphate, 150 mM NaCl, 1 mM DTT, 100 μg/ml BSA; 10 μl of partially purified GPI-PLC (T.b.) in 25 mM Tris/HCl (pH 8.0), 0.5 mM EDTA; 25 μl of crude GPI-PLD (heparinized rabbit serum) in 100 mM Tris/maleate (pH 7.4), 30 mM NaCl, 2 mM $CaCl_2$; 0.05 units of purified PI-PLC from *Staphylococcus aureus* (S.a.) in 20 mM Hepes/KOH (pH 7.0), 1 mM EDTA, 100 μg/ml BSA.

Enzyme assays. Published procedures were used for assaying lipoprotein lipase (LPL) (42, 47), 5′-nucleotidase (5′-N) (48), adenylate cyclase (AC) (49), and AChE (50) activities. The activity of alkaline phosphatase (aP) was determined from the rate of hydrolysis of *p*-nitrophenylphosphate (2 mg/ml) in 100 mM Tris/HCl (pH 9.2), 2 mM $MgCl_2$ at 30°C.

Aminopeptidase M (ApM) activity was tested using L-leucine-*p*-nitroanilide as a substrate. Samples were diluted 1:10 with 10 mM K,Na-phosphate buffer at pH 7.2 (900 μl) and adjusted to the desired detergent concentration. After incubation (30 min, 20°C) under stirring, samples were centrifuged (150,000*g*, 60 min, 4°C) and 450 μl of the supernatant was incubated (60 min, 37°C) with 50 μl of the substrate (0.2 μmol/100 μl water). After quenching with 500 μl 0.5% trifluoric acid and centrifugation, the hydrolysis rate was determined. For the determination of hydroxymethyl glutaryl coenzyme A-reductase (HMG-CoA-R) activity, 5 μl of microsomes was diluted to an appropriate working solution with buffer B (see preparation of rat liver microsomes) following a preincubation (10 min, 37°C) with 75 μl of an assay mix, containing 4.22 mg DTT, 13.2 mg $NADP^+$, 48.9 mg glucose 6-phosphate, 4.0 mg EDTA dissolved in 5.4 ml of a solution of 100 mM K_2HPO_4, 50 mM KCl (pH 7.4), and 60 μl of glu-

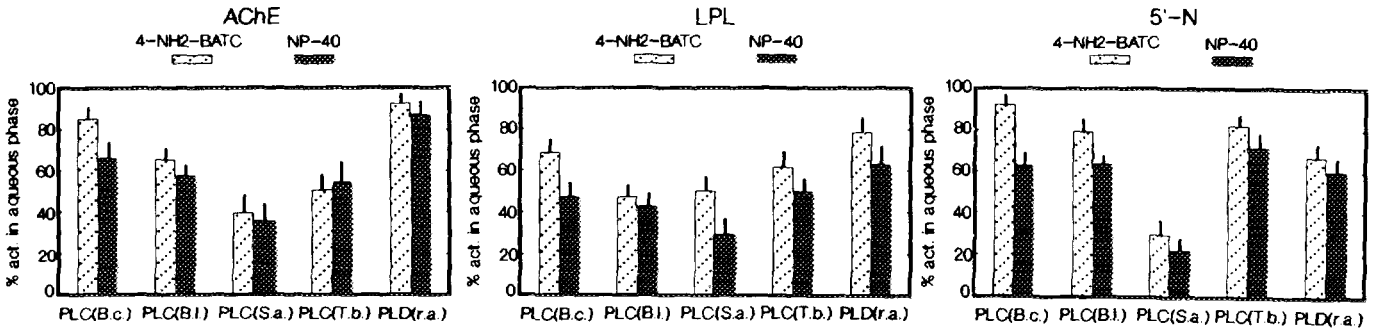

FIG. 7. Comparison of the cleavage efficiency of various (G)PI-PLs in the presence of BATC and NP-40. Rat adipocyte plasma membranes (LPL), bovine erythrocyte membranes (AChE), and rat liver plasma membranes (5'-N) were incubated with 1.5% BATC (striped bars) or 0.5% NP-40 (filled bars) and subsequently centrifuged. The supernatant was diluted 10-fold and incubated with various (G)PI-PLs (see Materials and Methods). The samples were subjected to TX-114 partitioning. The enzymatic activities of LPL, AChE, and 5'-N were determined before and after phase separation in the total mixture and aqueous phase, respectively, and are given as percentage total activity recovered in the aqueous phase (n = 4, ±SD).

cose-6-phosphate-dehydrogenase. The reaction was started by the addition of 20 μl of [^{14}C]HMG-CoA-solution, containing 117 μl of [^{14}C]HMG-CoA as supplied by the manufacturer (50 μCi in 2500 μl water, 58 mCi/mmol), 13 μl of a solution of 1.25 mg of unlabeled HMG-CoA in 100 μl of water, and 1170 μl of assay mix. After 15 min the reaction was terminated by the addition of 400 μl of a suspension of a 2 eq Dowex 50 WX4 in 1 eq 1 M HCl. The resulting mixture was incubated (30 min, 37°C) and put onto an anion-exchange column (AG1-X8, formiate form, 100–200 mesh). The column was eluted first with 500 μl water. The following 1.75-ml elution volume (water) was collected and counted for radioactivity.

Insulin binding to solubilized adipocyte plasma membranes. Samples containing various concentrations of detergent in 50 mM Hepes/KOH (pH 7.4), 150 mM NaCl, 10 mg/ml BSA were incubated with 50 μl of

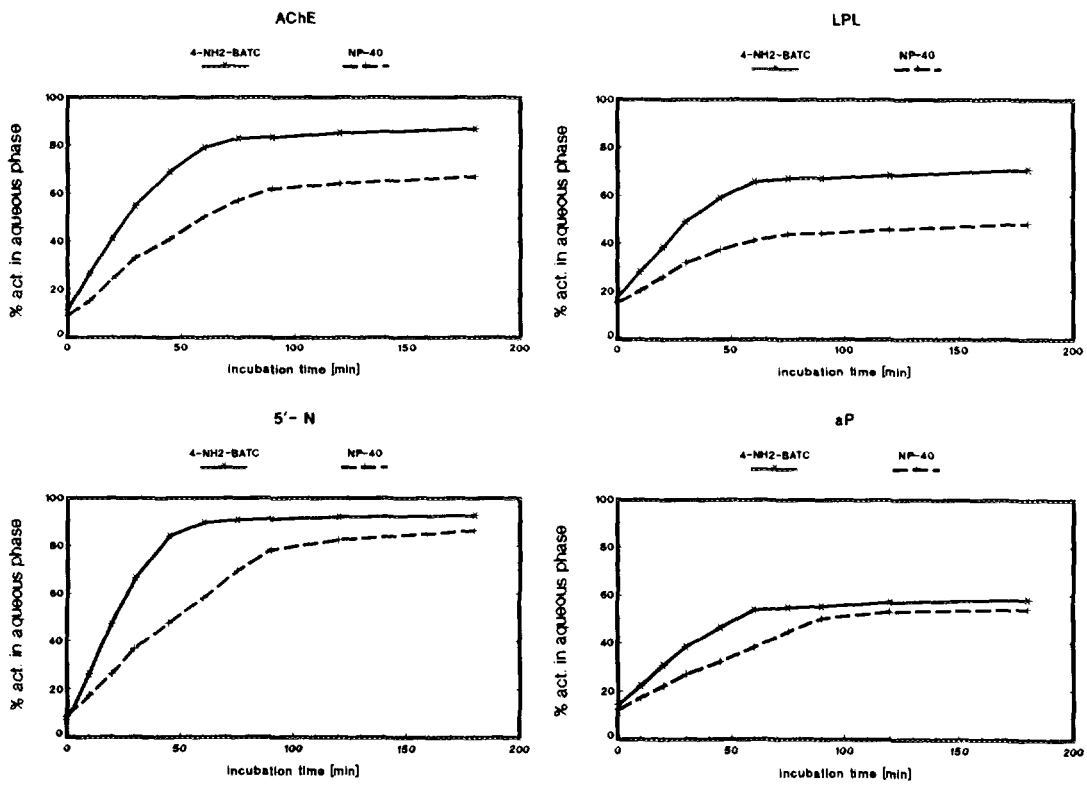

FIG. 8. Cleavage rate of PI-PLC is increased in the presence of BATC. Rat adipocyte plasma membranes (LPL), bovine erythrocyte membranes (AChE), and rat liver plasma membranes (5'-N, aP) were incubated with 1.5% BATC or 0.5% NP-40 and subsequently centrifuged. The supernatant was diluted 10-fold and incubated for various periods of time. The samples were subjected to TX-114 partitioning. The enzymatic activities were determined and calculated as described in the legend to Fig. 7.

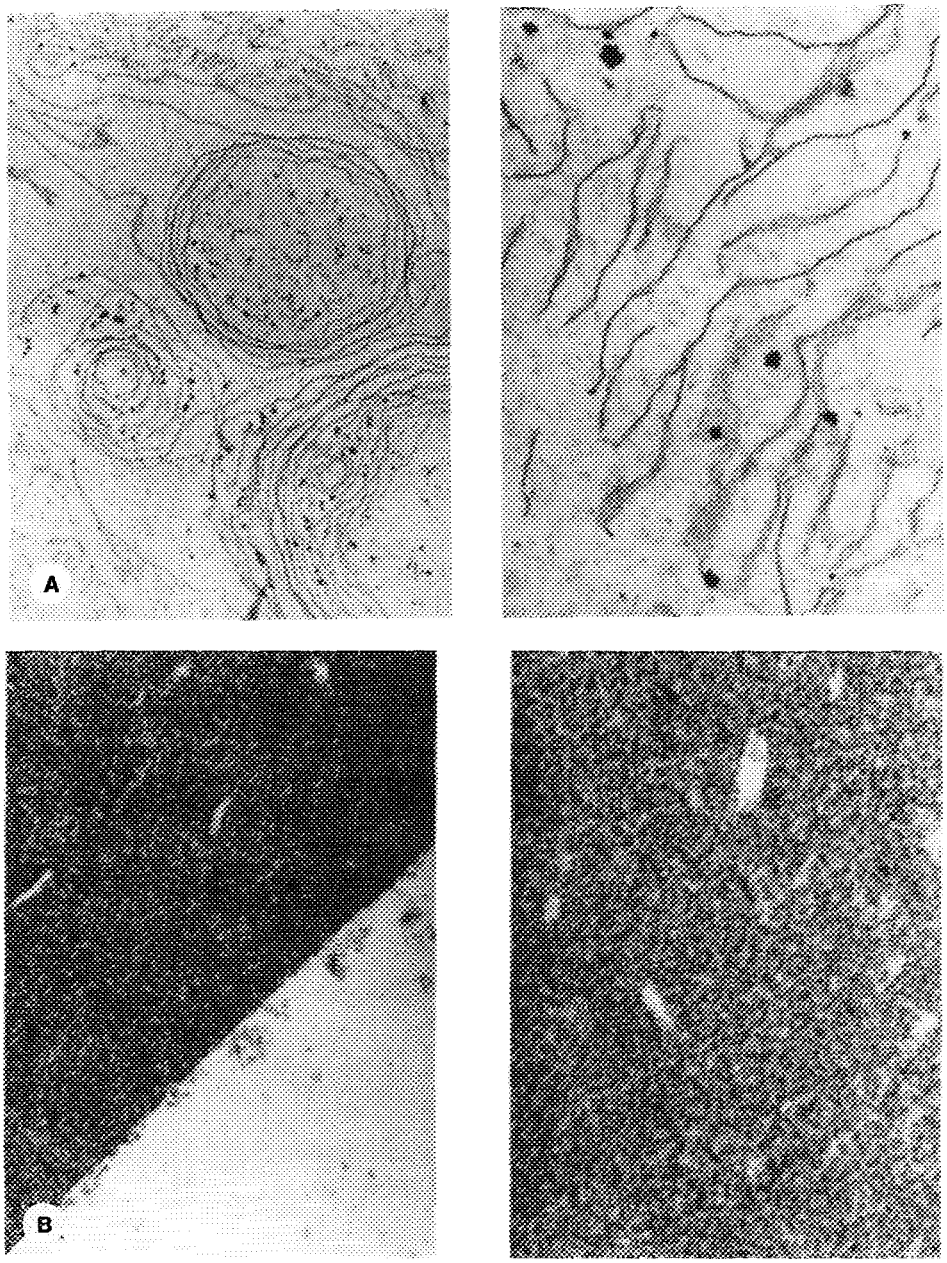

FIG. 9. (A) Rat liver plasma membranes were incubated (30 min, 20°C) and solubilized with BATC as described under Materials and Methods. The supernatant after centrifugation (2 h, 100,000*g*) was dialyzed (48 h, 4°C) against phosphate-buffered saline (pH 7.4). The resulting pellet after centrifugation (2 h, 300,000*g*) was fixed with glutaraldehyde and stained with osmium tetroxide. On the left side magnification is 10,000-fold and on the right 50,000-fold. Multilamellar vesicular structures which have formed due to BATC treatment are present. The heavily stained grains are presumably glycogen. (B) Rat liver plasma membranes were solubilized with NP-40 as described under Materials and Methods for 30 min at room temperature. Samples for analysis by electron microscopy were treated as described in (A). On the left side magnification is 50,000-fold and on the right, 100,000-fold.

^{125}I-labeled insulin (5×10^6 dpm/ml) in the absence or presence of 1 μM unlabeled insulin in a final volume of 200 μl (2 h, 20°C). Specific insulin binding was determined as described (51).

Miscellaneous procedures. Published procedures were used for SDS-PAGE using 5% stacking gels and 14% separating gels (52, 53), fluorography using ENHANCE and Kodak X-OMAT AR films, protein determination using the amidoblack staining method (54), and TX-114 partitioning (55) with the modifications described previously (42).

RESULTS

Solubilization of Membrane Proteins with BATC

In previous studies (31) it was shown that sodium 4'-amino-benzamido-taurocholate (Fig. 1) has pronounced selectivity for solubilization of certain membrane proteins. For instance, p3Ap3-synthetase from *Bacillus subtilis* was

enriched substantially compared to other membrane proteins. At that time, membrane proteins were classified as transmembrane proteins and peripheral membrane proteins. Since then, membrane anchorage by GPI structures was found for a considerable number of plasma membrane ectoproteins. Among them are aP (56) and 5'-N from mammalian tissues (57), AChE from mammalian blood cells (58), and LPL. In the latter case, anchorage by a covalently attached GPI moiety of at least a portion of the cell-surface-exposed LPL protein has been demonstrated for 3T3-L1 adipocytes (42, 59). These experiments do not exclude that a portion of LPL is anchored to the adipocyte plasma membrane via interaction with heparan sulfate proteoglycan releasable by heparin as has been shown by Chajek-Shaul and coworkers (70). However, heparan sulfate proteoglycan also occurs in a GPI-anchored form (60) and we found that 30–35% of the LPL activity from rat adipocytes can be released by heparin. 70–85% of the residual portion can be removed by subsequent incubation with bacterial PI-PLC (data not shown), suggesting that both types of membrane-anchored LPL are present in rat adipocytes.

We first studied the effect of BATC on extraction of GPI-proteins from different membrane preparations in comparison to octyl glucoside, one of the detergents most commonly used for solubilization of this class of proteins (20, 21). Plasma membranes from rat adipocytes (Fig. 2A) and bovine erythrocytes (Figs. 2B) were labeled metabolically with [^{35}S]methionine and then treated with detergent. The GPI-protein LPL and the transmembrane protein GLUT4 were immunoprecipitated from identical amounts of solubilized protein with specific antibodies and protein A–Sepharose (Fig. 2A), whereas GPI-anchored AChE and transmembrane GLUT1 were detected by immunoblotting and autoradiography (Fig. 2B). The results obtained for LPL by this experimental design reflect in part the characteristics of the GPI-anchored heparan sulfate proteoglycan. However, this fact does not devaluate our conclusions with regard to the specific enrichment and cleavage of GPI-proteins. Fluorography of the SDS–PAGE of both experiments demonstrates that for octyl glucoside the ratio of solubilized LPL/GLUT 4 and AChE/GLUT 1 remains constant over the entire detergent concentration range used (Fig. 2A, lower panel). However, the results obtained with BATC showed that the ratio of GPI-proteins and transmembrane proteins varies with the concentration and is highest between 0.05 and 0.2% (Fig. 2A, upper panel). This leads to a considerable specific enrichment of LPL and AChE toward total membrane protein. Under these conditions 60% (LPL)–85% (AChE) of the total GPI-protein contained in the corresponding membrane fraction (as evaluated by complete extraction with 2% SDS, see outer lanes) was solubilized by BATC. Immunoprecipitation from identical volumes after solubilization validated the concentration range for the selective effect of BATC and additionally proved the high extraction efficiency for GPI-proteins in comparison with SDS-solubilized total membranes (data not shown).

The potency of BATC in differential solubilization of GPI-proteins toward transmembrane proteins is compared with commercially available detergents, e.g., octyl glucoside, Chaps, deoxycholate, and TX-100, in Table I. Enzyme activity was measured in the supernatant after centrifugation of the detergent-treated membranes and was compared to the activity found in the total solubilisate. The results indicate that introducing BATC led to a 8- to 14-fold increase in the relative specific activities of GPI-proteins (LPL, AChE, aP, 5'-N). In case of transmembrane proteins (IR, GLUT4, AC, ApM, HMG-CoA-R), no specific enrichment was obtained with respect to the total protein concentration. With increasing concentrations of BATC and octyl glucoside, the total activity of AC, ApM, HMG-CoA-R, the total specific binding of IR, and the amount of immunoreactive GLUT 4 increased in the supernatant of solubilized and centrifuged membranes.

Cleavage of GPI-Proteins Using BATC Solubilized Membranes

The most widely used method for the identification of GPI-proteins is their accessibility for specific cleavage by (G)PI-PLC/D. Furthermore, some GPI-ectoproteins in different cell types, e.g., heparan sulfate proteoglycan (60), aP (61), LPL (59), 5'-N (62), and a cAMP-binding ectoprotein (63), which may originate from degradation of their hydrophobic membrane anchors by cellular anchor-specific PLs, have been identified in amphiphilic and hydrophilic form. Up to now, only a few (G)PI-PLs with different cleavage specificities (C and D) have been described. Their cleavage efficiency depends on the type and concentration of the detergent used for solubilization of GPI-protein.

Therefore, we evaluated BATC for its compatibility with the cleavage activity of some (G)PI-PLs. The effect of BATC solubilization on cleavage efficiency of PLs was tested as follows: plasma membrane from [^{35}S]methionine-labeled rat adipocytes were solubilized with 0.1% BATC or NP-40, respectively. After centrifugation, the supernatant was diluted and adjusted to the final concentration of the detergent. Cleavage was performed by incubation of the solubilized proteins with PI-PLC (B.c.). TX-114 partitioning of the samples and immunoprecipitation of LPL from both phases followed. Fluorography of the SDS–PAGE showed that the ratio of hydrophilic to amphiphilic LPL (60 kDa) reached its maximum at 0.15–0.2% BATC (Fig. 3A). Furthermore, analysis of anti-LPL immunoprecipitates clearly revealed that the resulting pattern in the detergent phase is a negative print of the pattern in the aqueous phase. The maximal cleavage rate of LPL by PI-PLC (B.c.) in presence

of NP-40 was lower and achieved at lower concentrations of detergent (0.075–0.1%) compared to BATC (Fig. 3B). Similar results were obtained using membranes from bovine erythrocytes and immunoblotting of BATC-solubilized AChE. Chaps and octyl glucoside were tested in comparison to BATC in analogous experiments and revealed no superior effect to either NP-40 or BATC (data not shown). Quantification of cleavage efficiency was performed by incubation of solubilized GPI-proteins from rat adipocyte plasma membranes or bovine erythrocyte membranes with PI-PLC (B.c.) in the presence of various concentrations of BATC or NP-40 (Fig. 4). After TX-114 partitioning, enzyme activity of AChE and LPL was measured in the aqueous phase and calculated as percentage of total solubilized GPI-protein activity before phase separation.

Compared to NP-40, BATC is more efficient in supporting lipolytic cleavage over almost the entire concentration range. Depending on the substrate, the effect of BATC varies between 70 (LPL) and 85% (AChE). Higher concentrations of both detergents significantly inhibited lipolytic cleavage. Other detergents, e.g., octyl glucoside, deoxycholate, Chaps, TX-100, and Mega-10 were also evaluated for their influence on cleavage efficiency. The activity of particular enzymes (LPL, AChE, 5′-N, aP) after treatment with PI-PLC (B.c.) was determined in the aqueous phase after TX-114 partitioning (Fig. 5). With regard to the GPI-proteins tested, best results can be obtained using BATC at concentrations proved optimal for solubilization. Consequently, the same concentration of BATC can be used for selective solubilization and lipolytic cleavage. In contrast, NP-40 supports cleavage of GPI membrane anchors best at concentrations (0.05–0.2%) at which no pronounced selectivity for extraction of GPI-proteins from plasma membranes can be detected. Cleavage specificity of PI-PLC (B.c.) in the presence of detergent was determined as follows: *myo*-[^{14}C]inositol- or [^{14}C]stearic acid-labeled rat adipocyte plasma membranes were incubated in presence of detergent (BATC or NP-40) and PI-PLC (B.c.) (Fig. 6). After PI-PLC (B.c.) cleavage, the hydrophilic and immunoprecipitated form of LPL retained the *myo*-inositol radiolabel (upper panel). Thus, this experimental design allows identification of exclusively the GPI-anchored form of LPL. No differences in cleavage efficiency of PI-PLC (B.c.) were observed for both detergents. Furthermore, the decrease of the amphiphilic and immunoprecipitated form of LPL, still containing the stearic acid radiolabel correlated well with the appearance of the hydrophilic, [^{14}C]inositol-labeled form of LPL and consequently was most pronounced at concentrations of maximal cleavage efficiency (lower panel). This demonstrated that the site of PI-PLC cleavage is located within the GPI-moiety of LPL. A subsequent second immunoprecipitation with anti-CRD antibodies was used to discriminate between PLC and PLD activity, since anti-CRD antibodies react primarily with the inositol-1,2-cyclic phosphate moiety generated by PI-PLC action (6, 16). The close correlation in the amounts of *myo*-[^{14}C]inositol-labeled total hydrophilic LPL (upper panel) and hydrophilic LPL recognized by anti-CRD antibodies (middle panel) indicated that most of the LPL molecules cleaved by PI-PLC (B.c.) in the presence of BATC and NP-40 at any concentration retained the inositol-1,2-cyclic phosphate residue. This strongly suggested that the cleavage specificity of PI-PLC (B.c.) is not altered by BATC even at high concentrations. In addition, it confirms that by using optimal concentrations of BATC (0.1–0.5%), cleavage efficiency is significantly higher compared to that with NP-40. Similar results were obtained for cleavage of the cAMP-binding ectoprotein from rat adipocyte or yeast plasma membranes (data not shown).

The influence of BATC and NP-40 on the enzymatic activity of (G)PI-PLs of different origin and specificity was determined as well. Cleavage efficiencies are strongly dependent on the PL used and are generally higher with BATC than with NP-40 (Fig. 7). The difference between both detergents is most significant in the case of PI-PLC (B.c.). Cleavage kinetics of four different GPI-proteins as model substrates for PI-PLC (B.c.) revealed that the initial cleavage velocity is increased significantly compared to that with NP-40 (Fig. 8). Looking on the results obtained with AChE and LPL, it seems that the use of BATC improves the accessibility of the cleavage site within the GPI-moiety for (G)PI-PLs.

DISCUSSION

The results of the present study demonstrate that 4′-NH_2-7-benzamido-taurocholate has certain advantages compared to other detergents already used in membrane biochemistry, particularly in the analysis and purification of GPI-proteins. We show that BATC selectively solubilizes GPI-proteins with an optimal concentration of 0.05–0.2%. In addition, BATC supports efficient cleavage of GPI-proteins by (G)PI-PLC(D) of various origin within the same concentration range. There have been several reports that GPI-proteins are solubilized poorly by nonionic detergents such as TX-100 (64, 65), especially at low temperature. Hooper and Turner (21) examined nine kidney microvillar enzymes and found that the transmembrane proteins were all solubilized completely by TX-100, while all of those anchored by GPI resisted solubilization (21). Furthermore, it has been reported that cellular glycosphingolipids and sphingomyelin are insoluble in TX-100 (66) and that GPI-proteins form defined complexes together with glycosphingolipids which are clustered in the plasma membrane (67). These mixed membrane patches may be involved in sorting of GPI-proteins to the apical surface in polarized cells (68) and the formation of plasma membrane caveolae for clathrin-independent endocytosis (71). Until now, the detergent that solubilized GPI-proteins most efficiently was octyl glucoside.

Consequently, the ratio of activity of ectoenzymes solubilized by octyl glucoside to that solubilized with TX-100 was used as a criterion for differentiation between GPI-proteins and proteins with a transmembrane polypeptide anchor (20, 21). In general, this ratio is greater than 1.60 for GPI-proteins and in the range of 0.95–1.30 for transmembrane proteins. These differences in the ratio between the two classes of membrane proteins are considerably lower than the enrichment factors for BATC-solubilized GPI-proteins versus transmembrane proteins. The structure of octyl glucoside is similar to that of a glycolipid, having a sugar as a polar head group. This structural feature might allow octyl glucoside to associate with glyco(sphingo)lipid-complexes and to solubilize them more efficiently than other detergents. Another explanation would be that only those detergents with a CMC as exhibited by octyl glucoside are capable of solubilizing substantial amounts of GPI-proteins. However, preliminary experiments suggest that the CMC of BATC is surprisingly low (45 μM) and the number of molecules per micelle (approx. 70) is relatively high (S. Müllner, unpublished results). Based on the opposing physical properties of BATC and octyl glucoside, the latter detergent may solubilize GPI-protein/glycosphingolipid complexes as a whole, while BATC may disintegrate those complexes by forming mixed micelles preferentially with GPI-proteins. In order to provide total disintegration, it is known that detergents with relatively low CMC are needed (69). BATC, NP-40, and TX-100, but not octyl glucoside, exhibit this respective feature. Comparison of the performance of NP-40 and BATC by analyzing the solubilized material with an electron microscope gives further evidence for this aspect. Multilamellar structures can be obtained after treatment of rat hepatocyte plasma membranes with BATC (Fig. 9A). In contrast, NP-40 treatment of the same membrane fraction yielded total amorphic material (Fig. 9B). In conclusion, our hypothesis for preferential solubilization of GPI-proteins from transmembrane proteins by the use of BATC is that it exhibits strong interaction with both, the glycan groups of glyco(sphingo)lipids and GPI-protein membrane anchors, but less affinity to other membrane components as well as hydrophobic protein domains. We think that the data provided prove the unique features of BATC; however, only a detailed evaluation by physical and physicochemical methods will be able to explain the action of BATC at the molecular level.

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