The sulphonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma-membrane proteins from 3T3 adipocytes

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Sulphonylurea drugs stimulate glucose transport and metabolism in muscle and fat cells *in vitro*. The molecular basis for the insulin-mimetic extrapancreatic effects of these oral antidiabetic therapeutic agents is unknown at present. Here we demonstrate that incubation of 3T3 adipocytes with the novel sulphonylurea, glimepiride, causes a time- and concentration-dependent release of the glycosylphosphatidylinositol (GPI)-anchored ectoproteins, 5′-nucleotidase, lipoprotein lipase and a 62 kDa cyclic AMP (cAMP)-binding protein from the plasma membrane into the culture medium. The change in the localization is accompanied by conversion of the membrane-anchored amphiphilic proteins into their soluble hydrophilic versions, as judged by pulse–chase experiments and Triton X-114 partitioning, and by appearance of anti-cross-reacting determinant (CRD) immunoreactivity of the released proteins as shown by Western blotting. Metabolic labelling of cells with *myo*-[^{14}C]inositol demonstrates that inositol is retained in the major portion of released lipoprotein lipase and cAMP-binding ectoprotein. The identification of inositol phosphate after deamination of these proteins with nitrous acid suggests cleavage of their GPI membrane anchor by a GPI-specific phospholipase C. However, after longer incubation with glimepiride the amount of soluble versions of the GPI-proteins lacking inositol and anti-CRD immunoreactivity increases, which may be caused by additional drug-stimulated hydrolytic events within their GPI structure or C-termini. Since insulin also stimulates membrane release of these GPI-modified proteins, and in combination with glimepiride in a synergistic manner, sulphonylurea drugs may exert their peripheral actions in adipose tissue by using (part of) the insulin postreceptor signalling cascade at the step of activation of a GPI-specific phospholipase C.

INTRODUCTION

For several decades sulphonylurea compounds have been widely used as hypoglycaemic agents in the treatment of type II or non-insulin-dependent diabetes mellitus. These drugs have been shown to act at both pancreatic and extrapancreatic loci [for a review see Boyd (1988) and Gerich (1989)]. The initial effects of sulphonylurea administration appear to arise from acute stimulation of the rate of insulin release from the pancreas (Yalow et al., 1960).

Recently, attention has been focused on the extrapancreatic actions of sulphonylureas, since prolonged drug studies with diabetic patients have demonstrated that plasma insulin levels often return to pretreatment levels even though the sulphonylurea-induced hypoglycaemic effect persists (Simonson et al., 1970, 1984; Lebovitz et al., 1977; Greenfield et al., 1982; Kolterman et al., 1984; Mandarino and Gerich, 1984; Ward et al. 1985). Thus these compounds possess the interesting additional property of increasing glucose uptake and utilization in peripheral tissues after prolonged exposure (Feldman and Lebovitz, 1971). Similar findings have been reported in studies with animals exposed for prolonged intervals to various sulphonylurea agents (Caren and Corbo, 1957; Putnam et al., 1981; Hirshman and Horton, 1990). It remains a matter of debate as to whether the improved insulin sensitivity observed during studies *in vivo* is due to improved overall metabolic control or a direct site of action of the drug at the muscle or fat cell.

To discriminate between direct sites of action of sulphonylurea drugs and effects mediated via insulin, experiments *in vitro* with isolated cell preparations are of advantage. During the last two decades a large body of evidence has accumulated that sulphonylurea agents enhance basal and insulin-stimulated glucose transport and metabolism in both human and animal muscle (Gibson et al., 1980; Rogers et al., 1987; Wang et al., 1987, 1989) and fat cells (Jacobs et al., 1989; Farese et al., 1991) *in vitro*. However, the molecular mechanisms for these stimulatory effects of the drugs are unknown. The increased insulin sensitivity *in vitro* may be caused by a higher number, binding affinity or tyrosine kinase activity of the insulin receptor. However, the evidence available strongly points toward a post-receptor effect (Maloff and Lockwood, 1981; Davidson and Sladen, 1987; Jacobs et al., 1987; Bak et al., 1989), which would also explain the stimulation of glucose transport in the absence of insulin.

The activation of protein kinase C via a sulphonylurea-induced increase in diacylglycerol during stimulation of basal glucose transport in cultured myocytes and adipocytes has been suggested (Rogers et al., 1987; Farese et al., 1991; Davidson et al., 1991). One potential source of diacylglycerol may reside in glucosylphosphatidylinositol (GPI) membrane anchors of certain plasma-membrane ectoproteins, since their metabolism in response to

Abbreviations used: cAMP, cyclic AMP; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein lipase; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; CRD, cross-reacting determinant; PI, phosphatidylinositol; GPI, glycosylphosphatidylinositol; PI/GPI-PLC, PI/GPI-specific phospholipase C; GPI-protein, protein that is synthesized with a GPI modification, irrespective of whether this structure is preserved or not (e.g. after lipolytic cleavage).

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insulin accompanied by release of the protein moiety from the cell surface of adipocytes and myocytes has been observed (Chan et al., 1988; Romero et al., 1988; Lisanti et al., 1989) and correlated with stimulation of glucose metabolism (Romero et al., 1988). Furthermore, the cleavage products of structurally related free GPI lipids, diacylglycerol and polar phosphoinositol-glycan headgroups, activate glucose transport in isolated rat adipocytes presumably by a two-step mechanism (Obermaier-Kusser et al., 1989; Vogt et al., 1990).

Therefore we have studied whether degradation of the GPI membrane anchor and membrane release of GPI-proteins are stimulated by sulphonylurea drugs.

MATERIALS AND METHODS

Materials

myo-[1-^{14}C]Inositol (55 mCi/mmol), glycerol tri[9,10(*n*)-^{3}H] oleate (0.6 Ci/mmol), [2,8-^{3}H]cAMP (45 Ci/mmol), *myo*-L-[U-^{14}C]inositol 1-phosphate (50 mCi/mmol) and goat anti-(rabbit IgG) coupled to peroxidase (affinity-purified) were bought from Amersham-Buchler (Braunschweig, Germany); 8-N_3-[^{32}P]cAMP (20 Ci/mmol), 8-N_3-[^{3}H]cAMP (20 Ci/mmol), L-[U-^{14}C]leucine (300 mCi/mmol) and L-[4,5-^{3}H(n)]leucine (5 Ci/mmol) were from ICN (Eschwege, Germany); [G-^{3}H]AMP (25 Ci/mmol) and EN^{3}HANCE was purchased from DuPont–New England Nuclear (Dreieich, Germany); 3T3 fibroblasts clone A31 were obtained from the American Type Culture Collection (CCL 163). Phenylmethanesulphonyl fluoride (PMSF), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, defatted BSA and phosphatidylcholine were provided from Sigma (Deisenhofen, Germany); Dulbecco's minimal essential medium (DMEM), Hanks buffer, antibiotics and sera for cell culture, pertussis and cholera toxin were from Gibco-BRL (Eggenstein-Leopoldshafen, Germany); Protein A–Sepharose and N^6-(2-aminoethyl)-cAMP–Sepharose were purchased from Pharmacia (Freiburg, Germany); PI-specific phospholipase C (PI-PLC) (*Bacillus cereus*), cycloheximide, leupeptin and antipain hydrochloride were provided from Boehringer (Mannheim, Germany); pepstatin A was bought from Fluka (Neu-Ulm, Germany); α_2-macroglobulin, aminoethylbenzylsulphonyl-fluoride, silica gel Si60 t.l.c. plates and all other chemicals (highest purity available) were obtained from Merck, Darmstadt, Germany. A polyclonal rabbit antiserum against purified human milk lipoprotein lipase (LPL) was kindly provided by Dr. C.-S. Wang (Oklahoma, OK, U.S.A.). Polyclonal anti-[cross-reacting determinant (CRD)] antibodies raised against purified soluble variant surface glycoprotein from MITat 1.2 in New Zealand White rabbits were a kind gift from P. Overath, Tübingen, Germany. Semi-synthetic human insulin and glimepiride were prepared by the Pharma Synthesis Department of Hoechst Aktiengesellschaft, Frankfurt. Glimepiride was dissolved in 25 mM Hepes/KOH (pH 7.4) to give a 2 mM stock solution made daily.

Cell culture

Cell culture was performed according to published procedures (Chan et al., 1988; Harrison et al., 1991a, b). Briefly, 3T3 fibroblasts were seeded at a density of 60 cells/mm^2, grown in DMEM containing 25 mM glucose, 10 % fetal calf serum (FCS), 50 units/ml penicillin and 50 μg/ml streptomycin sulphate (20 ml of medium per culture flask or 1.5 ml per well of a 24-well culture plate) and maintained in a 10 % CO_2-humidified atmosphere at 37 °C. The medium was changed every 2–3 days. Several days after the cells had achieved confluence, differentiation was induced with the same volume of DMEM containing 10 % FCS, 10 μg/ml insulin, 0.25 μM dexamethasone, 0.5 mM IBMX, 50 units/ml penicillin and 50 μg/ml streptomycin sulphate. After 2 days, the cells were incubated with the same medium without IBMX and dexamethasone for 2 additional days. Subsequently the cells were maintained in DMEM containing 10 % FCS and antibiotics. The medium was changed at intervals of 2–3 days. Cells were used 5–10 days after the induction of differentiation. About 80–90 % of these cells expressed the adipocyte phenotype. Living cell numbers were determined by counting the Trypan Blue-excluding cells harvested after trypsin treatment (0.1 % trypsin for 10 min at 30 °C) of the cell monolayers by using a haemocytometer.

Cell membrane preparation

Plasma membranes from 3T3 adipocytes were prepared as described by Simpson et al. (1983) with modifications according to James et al. (1989) and Clancy and Czech (1990). Briefly, cells were grown in culture flasks and incubated in 20 ml of high-glucose (25 mM) DMEM containing 10 % FCS and antibiotics with or without agent as indicated at 37 °C for the times indicated. Subsequently, the cells were washed once with 25 ml of room temperature phosphate-buffered saline/1 mM EDTA/250 mM sucrose and twice with 15 ml of buffer I (25 mM Hepes, 1 mM EDTA, 1 mM PMSF, 250 mM sucrose, pH 7.4). The monolayer from one flask was scraped into 50 ml of ice-cold buffer I supplemented with 0.1 mM PMSF and immediately homogenized with 10 strokes of a motor-driven (750 rev./min) Teflon pestle in a 60 ml glass homogenizer (Braun, Melsungen, Germany). The homogenate was centrifuged (16000 *g*, 20 min, 4 °C). The pellet was resuspended in 10 ml of buffer II (20 mM Hepes, 1 mM EDTA, pH 7.4) with a Dounce homogenizer and layered on to 20 ml of a sucrose cushion (1.12 M sucrose in buffer II) and centrifuged (100000 *g*, 1 h, 4 °C). Plasma membranes were recovered from the top of the sucrose cushion, resuspended in 30 ml of buffer II and collected by centrifugation (30000 *g*, 30 min, 4 °C). The plasma membranes were washed by repetition of the last centrifugation and finally resuspended in buffer II containing 1 mM PMSF at about 2 mg of protein/ml.

Metabolic labelling

The labelling was performed in culture flasks as described by Chan et al. (1988) with modifications. Cells were washed with 20 ml of Hanks' buffer (pH 7.4), 0.5 % BSA and incubated with 250 μCi of *myo*-[^{14}C]inositol in 20 ml of DMEM containing 10 % FCS, 5 mM glucose, 0.5 % BSA, 50 units/ml penicillin and 50 μg/ml streptomycin sulphate for 14 h in 5 % CO_2/95 % air at 37 °C. For labelling with radioactive leucine the cells were incubated with 0.75 mCi [^{14}C]leucine or 0.1 mCi [^{3}H]leucine in leucine-free DMEM (see above). Subsequently, the cells were washed three times with 10 ml of Hanks' buffer (pH 7.4)/0.5 % BSA/1 mM *myo*-inositol or 10 mM leucine and then for incubation with glimepiride (chase) supplemented with 20 ml of high-glucose DMEM/10 % FCS/0.5 % BSA/0.1 mM *myo*-inositol or leucine/50 units/ml penicillin/50 μg/ml streptomycin sulphate/1 mM IBMX/0.1 mM PMSF.

Photoaffinity labelling

This was carried out by a method adapted from one used for yeast cells (Müller and Bandlow, 1991a). The labelling was

performed in 24-well culture plates. Cells were washed twice with 2 ml of DMEM containing 1% BSA, 1 mM EDTA, 100 mM NaCl and without glucose, serum and antibiotics. To each monolayer, 10 μCi 8-N_3-[^{32}P]cAMP (0.5 nmol) or 2 μCi 8-N_3-[^{3}H]cAMP in 0.5 ml of phosphate-buffered saline/250 mM sucrose/1 mM EDTA/50 mM KCl/10 mM $MgCl_2$/2 mM $MnCl_2$/1 mM IBMX/1 mM dithiothreitol (DTT)/1 mM AMP/0.1 mM PMSF was added. The wells were incubated for 30 min at 4 °C and then irradiated with u.v. light at 254 nm (8000 μW/cm^2) at a distance of 2 cm for 2 min. Subsequently, the cells were washed twice with 2 ml of the same buffer containing 1 mM unlabelled cAMP instead of 8-N_3-[^{32}P]cAMP and then for incubation with glimepiride (chase) supplemented with 1 ml of high-glucose DMEM, 0.1 mM cAMP, 10% FCS, 0.5% BSA, 50 units/ml penicillin, 50 μg/ml streptomycin sulphate, 1 mM IBMX and 0.1 mM PMSF.

Triton X-114 partitioning

This was carried as described by Bordier (1981) with the following modifications: 50 μl of the incubation mixture was added to 1 ml ice-cold Triton X-114 (2%) in 25 mM Tris/HCl (pH 7.4)/144 mM NaCl. The mixture was layered on to a cushion of 0.4 ml 250 mM sucrose/25 mM Tris/HCl (pH 7.4) on ice. Phase separation was induced by warming up to 37 °C and centrifugation (Microfuge, 2 min). The detergent phase was re-extracted twice. The aqueous phases were pooled.

Immunoprecipitation

Immunoprecipitation was performed according to Müller and Bandlow (1991b) with the following modifications. Medium samples (200 μl of a supernatant, Microfuge, 2 min) were passed through a Millex GV 0.2 μM filter, subsequently precipitated with 10% (v/v) trichloroacetic acid and dissolved in 50 μl of 10 mM Tris/HCl (pH 8.0)/1 mM EDTA/2% (w/v) SDS. After being heated at 95 °C for 5 min and centrifugation (Microfuge, 5 min), the supernatant was supplemented with 1 ml of immunoprecipitation buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Triton X-100). Membrane samples (50 μg protein in buffer II) were adjusted to 2% SDS and processed as described above. Then 10 μl of a 1:250 dilution of a polyclonal rabbit antiserum against human milk LPL was added. After 12 h at 4 °C followed by addition of 50 μl of Protein A–Sepharose, the incubation was continued for 2 h. The immunoprecipitates were collected by centrifugation, washed twice with immunoprecipitation buffer, twice with buffer not containing Triton X-100 and finally dissolved in 60 mM Tris/HCl (pH 6.8)/2% SDS/5% 2-mercaptoethanol/10% (v/v) glycine and analysed by SDS/PAGE and fluorography.

For immunoprecipitation with anti-CRD antibodies, cAMP affinity-purified samples were precipitated with poly(ethylene glycol) 4000 (see below), washed and dissolved in 1 ml of 20 mM Tris/HCl (pH 7.5)/0.5 mM EDTA/1 mM PMSF/0.1% SDS. Anti-LPL immunoprecipitates (material adsorbed to Protein A–Sepharose beads obtained after centrifugation, see above) and trichloroacetic acid precipitates (obtained after centrifugation and two acetone washes) were supplemented with 50 μl of 60 mM Tris/HCl (pH 6.8)/0.5 mM EDTA/1 mM PMSF/2% SDS/2% mercaptoethanol, incubated at 95 °C for 5 min and centrifuged (10000 *g*, 2 min). The pellet was washed three times with immunoprecipitation buffer and the combined supernatants were adjusted to 1 ml with immunoprecipitation buffer. The samples were incubated with 5 μl anti-CRD antiserum (1:500 dilution) for 1 h at 4 °C and then with 25 μl goat anti-(rabbit IgG) antibodies coupled to agarose for 30 min at 4 °C with gentle shaking. After centrifugation (10000 *g*, 2 min), the pellet was washed and processed as described above. Under these conditions the anti-CRD antibodies quantitatively immunoprecipitate PI-PLC-cleaved cAMP-binding protein and LPL (see Figure 1).

Affinity purification

This was carried out as described (Müller et al., 1992) with the following modifications: 200 μg of solubilized plasma-membrane protein (25 mM Mops/KOH, pH 7.0, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5% octyl glucoside) or 250 μl of culture medium were applied to a 2 ml column of N^6-(2-aminoethyl)-cAMP–Sepharose. The column was washed five times with 25 mM Mops/KOH (pH 7.2)/100 mM sodium citrate/150 mM NaCl/2 mM DTT/1 mM $MgCl_2$/10% glycerol/1 mg/ml BSA/1 mM IBMX and eluted with 2 ml of the same buffer containing 10 mM cAMP. The initial 250 μl of the eluate was desalted by centrifugation through a 1 ml column of Sephadex G-25 and then precipitated with 5% poly(ethylene glycol) 4000. The precipitates were dissolved in 20 mM Mops/KOH (pH 7.2)/0.5 mM EDTA/0.1 mM PMSF/0.5% octyl glucoside.

Digestion with PLC

The cell monolayer of one well (per 24-well plate) was washed with DMEM and then incubated with 0.5 ml DMEM containing 0.05 or 0.25 unit PI-PLC (*Bacillus cereus*) (1 unit is defined as 5 μmol bovine acetylcholinesterase cleaved/min), 120 mM NaCl, 2 mM EDTA, 5 mM DTT, 0.005% Triton X-100 and 0.1 mg/ml phosphatidylcholine for 2 h at 37 °C.

T.l.c.

The samples were concentrated by freeze-drying, suspended in water, applied to a silica gel Si60 t.l.c. plate and developed twice in pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.). Radioactive spots were visualized by autoradiography.

Enzyme assays

LPL activity was assayed as described (Nilsson-Ehle and Schotz, 1976) with a concentrated anhydrous emulsion of [^{3}H]glycerol tri[9,10(n)-^{3}H]oleate in glycerol stabilized with phosphatidylcholine as substrate. The final concentrations were triolein (7.5 mM), phosphatidylcholine (0.6 mM), BSA (0.75%, w/v), glycerol (10%) and FCS (7.5%, v/v) in 50 mM Hepes/KOH (pH 7.8). Incubations were carried out in a total volume of 250 μl for 20 min at 37 °C. The activity of 5′-nucleotidase was assayed as described by Avruch and Wallach (1971) with minor modifications. Briefly, the test was performed in a total volume of 1 ml containing 100 mM Tris/HCl (pH 7.4), 20 mM $MgCl_2$ and 0.5 mM [^{3}H]AMP (100 nCi) for 30 min at 37 °C. The reaction was stopped by the addition of 250 mM $ZnSO_4$. Unhydrolysed AMP and protein were precipitated by the addition of 250 μl of 0.3 M $Ba(OH)_2$. The adenosine remaining in solution after centrifugation of the sample (Microfuge, 2 min) was counted for radioactivity in 10 ml of scintillation cocktail (Zinsser 361). Adenylate cyclase was measured as described by Salomon et al. (1974). Lactate dehydrogenase was assayed by a standard spectrophotometric assay (Wroblewski and LaDue, 1955). Mg^{2+}-ATPase was determined as in Hidalgo et al. (1983). Succinate:cytochrome *c* oxidoreductase assay was carried out as described in Lang et al. (1977).

cAMP binding

This was measured by the methods of Pomerantz et al. (1975), Rödel et al. (1985) and Müller and Bandlow (1989) with modifications. Medium or membranes were precipitated with 8% poly(ethylene glycol) (15 min, 4 °C; Microfuge, 5 min) and dissolved in binding buffer (25 mM Mes, pH 6.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EDTA, 0.2 mM DTT, 1 mM IBMX, 0.1 mM PMSF, 0.1 mM AMP, 0.2% octyl glucoside). About 100 μg of protein was incubated with 100 nCi [^{3}H]cAMP (about 2 nmol) in the presence or absence of 0.8 mM cAMP in a total volume of 250 μl for 5 min at 4 °C. After addition of 0.5 ml ice-cold saturated $(NH_4)_2SO_4$, the sample was rapidly filtered through a nitrocellulose filter (Millipore, HAWP, 0.45 μM) under vacuum. The filter was washed three times with 5 ml of washing buffer (25 mM Mes, pH 6.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EDTA, 1 mM IBMX, 0.1 mM PMSF, 1 mM AMP), dried at 70 °C and counted for radioactivity in 5 ml of scintillation cocktail (Zinsser 361). Specific cAMP binding was calculated as the difference between radioactivity measured in the absence and presence of 0.8 mM cAMP.

Miscellaneous procedures

Nitrous acid deamination (Müller and Bandlow, 1991a), extraction from silica gel, elution of proteins from polyacrylamide gels (Müller et al., 1992), SDS/PAGE using 15% separating and 5% stacking gels in the presence of urea (Laemmli, 1970; Müller and Zimmermann, 1987), fluorography using EN^{3}HANCE (Chamberlain, 1989), densitometry with an LKB densitometer, protein determination (Popov et al., 1975; Bradford, 1976) and liquid-scintillation counting using scintillation cocktail ASC II (Amersham-Buchler) were performed according to published procedures.

RESULTS

3T3-L1 adipocytes contain a number of GPI-anchored plasma-membrane proteins (Chan et al., 1988; Lisanti et al., 1989). Release of these ectoproteins from the plasma membrane into the culture medium by removal of their GPI membrane anchor has been observed after short-term incubation with insulin (see the Discussion). We asked whether sulphonylurea drugs, which exert certain insulin-like effects on these cells, also mimic membrane release of GPI-proteins.

We studied three proteins for which anchorage at the plasma membrane by a GPI structure has been established, LPL (Chan et al., 1988), 5′-nucleotidase (Low and Finean, 1978) and a novel cAMP-binding ectoprotein (62 kDa). This protein can be detected by photoaffinity labelling with the membrane-impermeable 8-N_3-cAMP of intact adipocytes isolated by collagenase digestion under strictly controlled conditions or of 3T3 adipocytes cultured as monolayers but without trypsin treatment (Müller and Bandlow, 1991c; G. Müller and S. Wied, unpublished work). The presence of a GPI membrane anchor has been demonstrated by its metabolic labelling with typical constituents of this structure. A similar cAMP-binding protein has been identified in the yeast *Saccharomyces cerevisiae* (Müller and Bandlow, 1991a,b).

In addition, the anchorage to the membrane by a GPI moiety of the three proteins in the 3T3 adipocytes from the subclone used in this study was confirmed by incubation of intact adipocytes with bacterial PI-PLC (Figure 1). This caused a concentration-dependent conversion of the total membrane-bound amphiphilic LPL (Figure 1a) and cAMP-binding protein (Figure 1b) into their soluble hydrophilic versions as judged by centrifugation and Triton X-114 partitioning which correlated with the appearance of anti-CRD immunoreactivity of the solubilized GPI-proteins as demonstrated by immunoprecipitation with anti-CRD antibodies. The epitope recognized by anti-CRD antibodies primarily consists of the inositol 1,2-cyclic monophosphate residue generated by PLC cleavage of the GPI moiety (Zamze et al., 1988) and thus was available only in the lipolytically cleaved (lanes 18, 20, 22 and 24) but not in the intact form of LPL and cAMP-binding ectoprotein (lanes 13–17, 19, 21 and 23). The immunoprecipitation of the radiolabelled PI-PLC-cleaved GPI-proteins by anti-CRD serum was nearly quantitative. Furthermore, up to 75% of 5′-nucleotidase activity was released from plasma-membrane vesicles after treatment with bacterial PI-PLC in a concentration-dependent manner (results not shown).

We next studied whether treatment of 3T3 adipocytes with the novel sulphonylurea compound, glimepiride, causes an increase

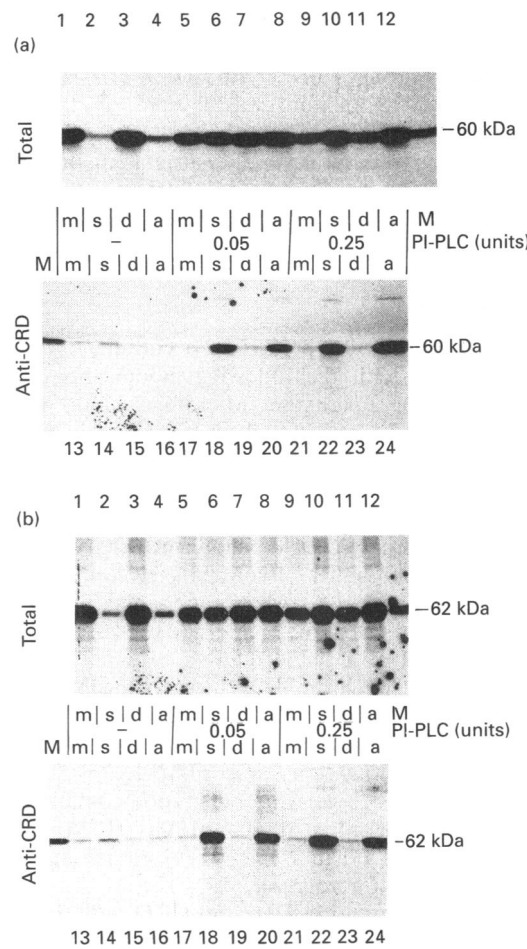

Figure 1 Susceptibility of GPI-proteins of 3T3 adipocytes to digestion with bacterial PI-PLC

3T3 adipocytes were metabolically labelled with *myo*-[^{14}C]inositol, incubated with different amounts of PI-PLC (*Bacillus cereus*) and then scraped from the plate. One-half of the total incubation mixture (culture medium and cells) was subjected to centrifugation (150000 ***g***, 1 h), the other half to Triton X-114 partitioning. The membrane (m) and soluble (s) fractions or detergent (d) and aqueous (a) phases were analysed by immunoprecipitation with anti-LPL serum (**a**, LPL) and cAMP–Sepharose affinity purification (**b**, cAMP-binding protein). One-half of the immunoprecipitates and affinity-purified material was subjected directly to SDS/PAGE and fluorography (upper gels, total), the other half was immunoprecipitated with anti-CRD antibodies and then analysed by SDS/PAGE and fluorography (lower gels, anti-CRD). Immunoprecipitated metabolically labelled LPL and N_3-[^{32}P]cAMP-photoaffinity-labelled cAMP-binding protein from adipocyte homogenate are shown as marker (M) on the margins.

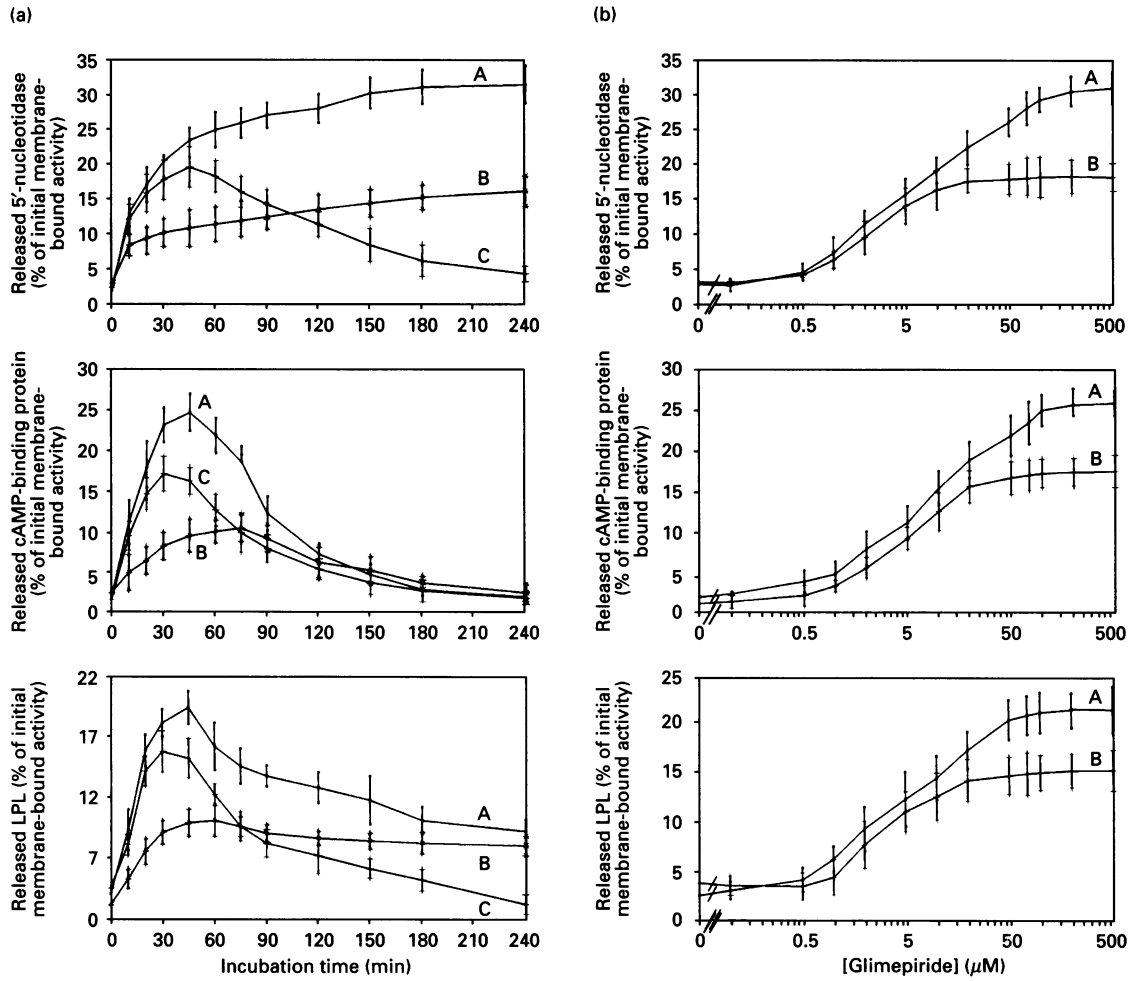

Figure 2 Glimepiride-induced increase in the activity of GPI-proteins in the culture medium

(**a**) Time course. 3T3 adipocytes were incubated in the absence (curve B) or presence of glimepiride (50 μM) without (curve A) or with (curve C) cycloheximide (150 μg/ml). After different times, portions of the culture medium were withdrawn and the activities of 5′-nucleotidase and LPL as well as cAMP-binding per ml of culture medium were measured. The total activity in the medium of one well was calculated as the percentage of the total activity determined in plasma membranes from the cells of one well at the zero time point in the absence of glimepiride which was set at 100% of initial membrane-bound activity. The corresponding specific activities of the medium samples were as follows: 5′-nucleotidase, 1.7 nmol/min per ml; LPL, 24.3 nmol/min per ml; cAMP-binding, 0.23 pmol/ml. The values represent the means of four different experiments (cell preparations) with triplicate measurements for each. (**b**) Concentration dependence. 3T3 adipocytes were incubated for 3 h with different concentrations of glimepiride in the absence (curve A) or presence (curve B) of cycloheximide (150 μg/ml). Portions of the culture medium were assayed for 5′-nucleotidase, LPL and cAMP-binding. The total activity recovered in the medium was calculated as the percentage of the initial membrane-bound activity as described above.

Table 1 Specific activities of GPI-anchored and transmembrane plasma-membrane enzymes in the presence or absence of glimepiride

3T3 adipocytes were incubated in the absence or presence of glimepiride (50 μM) without or with cycloheximide (150 μg/ml) for 2 h. After removal of the culture medium and washing of the cells, homogenate and plasma-membrane fractions were prepared. All samples were assayed for 5′-nucleotidase (expressed as nmol/min per mg), LPL (expressed as nmol/min per mg), cAMP-binding (expressed as pmol/mg), Mg^{2+}-dependent ATPase [expressed as nmol/min per mg or nmol/min per ml (medium)], adenylate cyclase [expressed as pmol/min per mg or pmol/min per ml (medium)], lactate dehydrogenase [expressed as μmol/min per mg or μmol/min per ml (medium)] and succinate (succ.):cytochrome (cyt.) *c* oxidoreductase [expressed as nmol/min per mg or nmol/min per ml (medium)]. The values are means of three different experiments (cell preparations), each with duplicate measurements.

	Homogenate		Plasma membrane		Medium	
	Control	Glimepiride	Control	Glimepiride	Control	Glimepiride
5′-Nucleotidase	3.5±0.5	2.0±0.4	58.3±4.5	12.7±1.1	see Figure 2	see Figure 2
LPL	1.9±0.3	0.6±0.2	25.7±4.6	6.7±0.8	see Figure 2	see Figure 2
cAMP-binding protein	n.d	n.d	2.6±0.4	0.4±0.2	see Figure 2	see Figure 2
Mg^{2+}-ATPase	277±38	298±49	1695±256	1578±227	6.7±1.1	8.9±2.2
Adenylate cyclase	0.4±0.1	0.6±0.2	2.9±0.7	2.4±0.6	n.d.	< 0.2±0.1
Lactate dehydrogenase	38.3±4.1	34.5±5.0	0.3±0.1	1.1±0.3	1.5±0.3	0.8±0.2
Succ.:cyt. *c* oxidoreductase	7.9±1.3	9.3±0.7	0.7±0.2	0.4±0.1	< 0.2±0.1	0.4±0.2

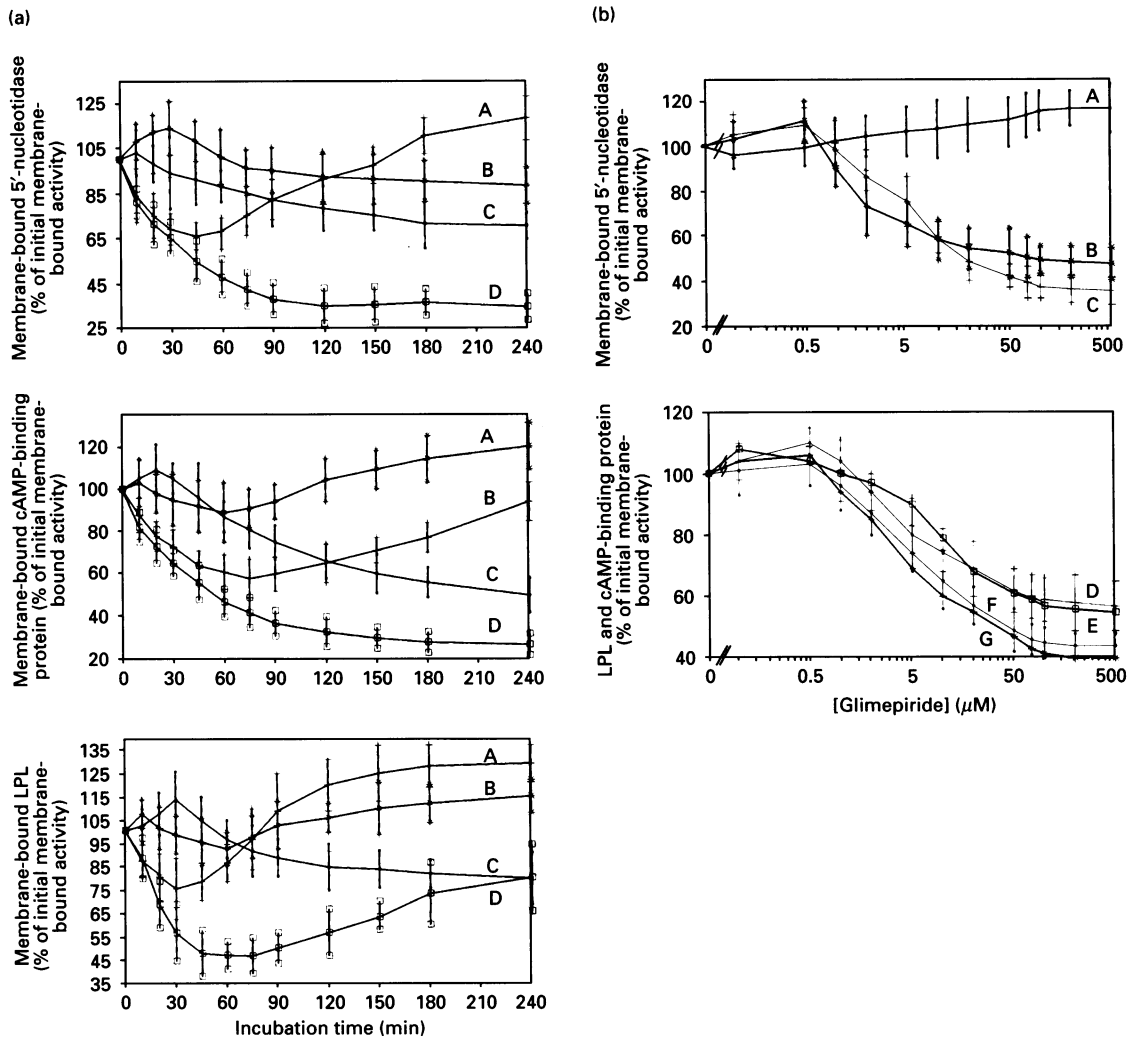

Figure 3 Glimepiride-induced decrease in the activity of GPI-proteins in the plasma membrane

(**a**) Time course. 3T3 adipocytes were incubated in the absence (curves B and C) or presence (curves A and D) of glimepiride (50 μM) without (curves A and B) or with (curves C and D) cycloheximide (150 μg/ml). After the times indicated, the culture medium was removed and the cells were washed and scraped off the plate. Plasma membranes were prepared and assayed for 5'-nucleotidase, LPL and cAMP-binding protein. The total activity determined in plasma membranes of the cells from one well was calculated as the percentage of the total plasma-membrane activity per well measured after the zero time point in the absence of glimepiride which was set at 100% of initial membrane-bound activity. The corresponding specific activities of plasma membranes were as follows: 5'-nucleotidase, 54.5 nmol/min per mg; LPL, 137.4 nmol/min per mg; cAMP-binding, 2.3 pmol/mg. The values represent the means of three different experiments (cell preparations) with triplicate measurements for each. (**b**) Concentration-dependence. 3T3 adipocytes were incubated with various concentrations of glimepiride in the absence (thick line) or presence of cycloheximide (150 μg/ml; thin line) for 30 min (curves B and C) or 4 h (curve A). 5'-Nucleotidase, LPL (curves D and E) and cAMP-binding (curves F and G) were determined. The total plasma-membrane activity was calculated as a percentage of the initial membrane-bound activity as described above.

in the activity of the GPI-proteins in the culture medium. For this the cells were incubated with glimepiride for various times before the medium was assayed for LPL, 5'-nucleotidase and cAMP-binding activity. Figure 2(a) shows that the total activities of all three GPI-proteins increased dramatically in the presence of glimepiride, but with different time courses, reached a plateau and, in the case of the LPL and cAMP-binding activity, decreased thereafter, reaching pretreatment levels after 2–4 h of incubation. This may be caused by inactivation (proteolysis) or adsorption to the cell surface (and subsequent endocytosis) of the released proteins. The loss of LPL activity in the presence of 3T3-L1 adipocytes is well known (Olivercrona and Semb, 1987).

The increase in enzyme activity in the culture medium was specific for GPI-proteins and not due to leakage of the plasma membrane, since it was not observed with the transmembrane plasma-membrane proteins, Mg^{2+}-ATPase and adenylate cyclase, or the cytosolic and mitochondrial proteins, lactate dehydrogenase and succinate:cytochrome *c* oxidoreductase respectively (Table 1). Control incubations (16 h) with glimepiride (500 μM) demonstrated at least 90% viability of the cells using Trypan Blue exclusion and overall protein synthesis (metabolic incorporation of [^{35}S]methionine into total cellular protein) as criteria (results not shown).

Interestingly, cycloheximide did not affect the initial increase but markedly decreased the maximal activity of the three GPI-proteins in the medium (Figure 2a, curve C). After 3 h of incubation with both glimepiride and cycloheximide, the activities fell below the starting values. (Under our experimental conditions, incubation with cycloheximide 10 min before addition of glimepiride decreased overall protein synthesis by more than 75%; results not shown.) These observations indicate that the time-dependent increase in enzyme activity in the medium

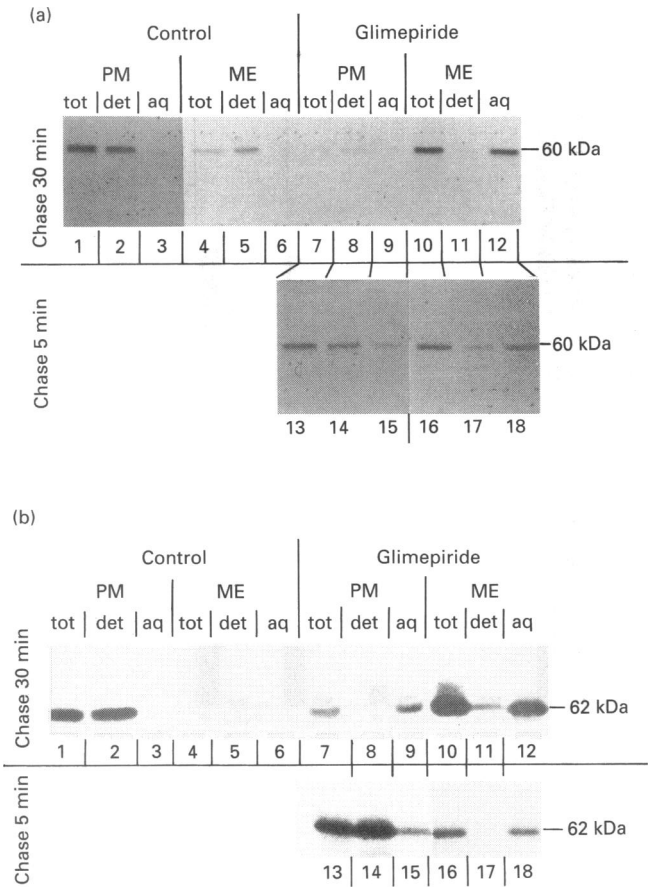

Figure 4 Conversion of GPI-proteins from the amphiphilic into the hydrophilic form

3T3 adipocytes were metabolically labelled with *myo*-[^{14}C]inositol (**a**; LPL) or photoaffinity-labelled with 8-N_3-[^{32}P]cAMP (**b**; cAMP-binding protein). The cells were washed and incubated in the absence (Control) or presence of glimepiride (50 μM) for 30 or 5 min (Chase). Subsequently, the culture medium (ME) was removed and concentrated by trichloroacetic acid precipitation (5% trichloroacetic acid, two washes with acetone). The cells were washed and plasma membranes (PM) were prepared. One-half of the culture medium and plasma membranes was partitioned into Triton X-114 (det) and aqueous (aq) phases; the other half was left (tot). For identification of LPL, each sample was immunoprecipitated and analysed by SDS/PAGE and fluorography. For visualization of the photoaffinity-labelled cAMP-binding protein each samples was applied directly to the gel. Molecular masses were derived from marker proteins run in parallel on the same gel.

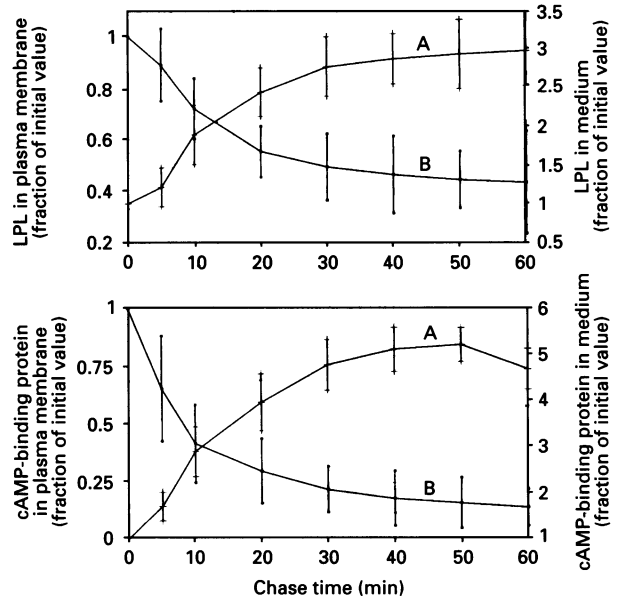

Figure 5 Time course for the amphiphilic/hydrophilic conversion of LPL (a) and cAMP-binding protein (b)

The experiment was carried out as described in the legend to Figure 3 in the absence or presence of glimepiride (50 μM) but with various chase times. Culture medium and plasma membranes were subjected to Triton X-114 partitioning. The aqueous phase recovered from the culture medium (curve A) and the detergent phase recovered from plasma-membrane samples (curve B) were analysed after immunoprecipitation (LPL) or directly (cAMP-binding protein) by SDS/PAGE and densitometry of the fluorogram. The ratio between the densitometric values for the plasma membrane (amphiphilic version) or culture medium (hydrophilic version) respectively, in the presence or absence of glimepiride, was calculated for each time point. For the zero time point this ratio was set at 1.0 (fraction of initial value of the plasma membrane and medium respectively). Each value represents the means of three different experiments using different cell preparations.

involves both the release from the cell surface, which is independent of protein synthesis, and synthesis and cell-surface expression of the GPI-proteins.

The concentration of glimepiride necessary for half-maximal increase in LPL, 5′-nucleotidase and cAMP-binding activity in the medium during the initial cycloheximide-insensitive phase was 7–10 μM (Figure 2b), with a maximal effect at 60–75 μM, and is unaffected by cycloheximide. This confirms that the initial increase in GPI-protein activity in the medium originates from preformed molecules and not from proteins synthesized *de novo* and expressed at the cell surface in response to the drug. However, stimulation of the expression of GPI-protein genes during long-term treatment with sulphonylureas, as has been found for certain glucose-transporter isoforms (Wang et al., 1989; Tordjman et al., 1989), is not excluded.

If the above hypothesis is true, treatment of cells with glimepiride in the presence of cycloheximide should result in depletion of GPI-proteins at the cell surface. To test this, the total activities of the GPI-proteins in plasma membranes isolated from glimepiride-stimulated 3T3 adipocytes were determined. The method used for the fractionation of the cultured fat cells yielded plasma membranes of sufficient purity, as demonstrated by an up to 10-fold enrichment in the plasma-membrane proteins, adenylate cyclase and Mg^{2+}-ATPase, together with the GPI-proteins, and the diminution of mitochondrial and cytosolic proteins (Table 1). Figure 3(a) shows that, in the absence of cycloheximide, glimepiride provoked an initial rapid decrease in plasma medium activity of each GPI-protein followed by a rapid increase, finally reaching pretreatment or even higher levels. In the absence of glimepiride the specific activities remained constant. However, the presence of cycloheximide caused a prolonged phase of decrease in the plasma-membrane activity and inhibited its recovery. Incubation with cycloheximide alone caused a slight decrease in activity during the entire incubation period, indicating a basal turnover (degradation and/or release) of the GPI-proteins studied. The altered distribution of GPI-protein but not of integral membrane protein activity (Table 1) again emphasizes the selectivity of the sulphonylurea-stimulated releasing process.

The drug concentrations required for maximal loss of GPI-protein activity in the plasma membrane were in the range 3–8 μM (Figure 3b, thick lines). Cycloheximide had only minor effects (Figure 3b, thin lines) in agreement with the acute effects of glimepiride. However, after long-term incubation with any concentration of glimepiride, pretreatment levels of plasma-membrane activity were measured in the absence of cycloheximide (shown only for 5′-nucleotidase in Figure 3b), indicating

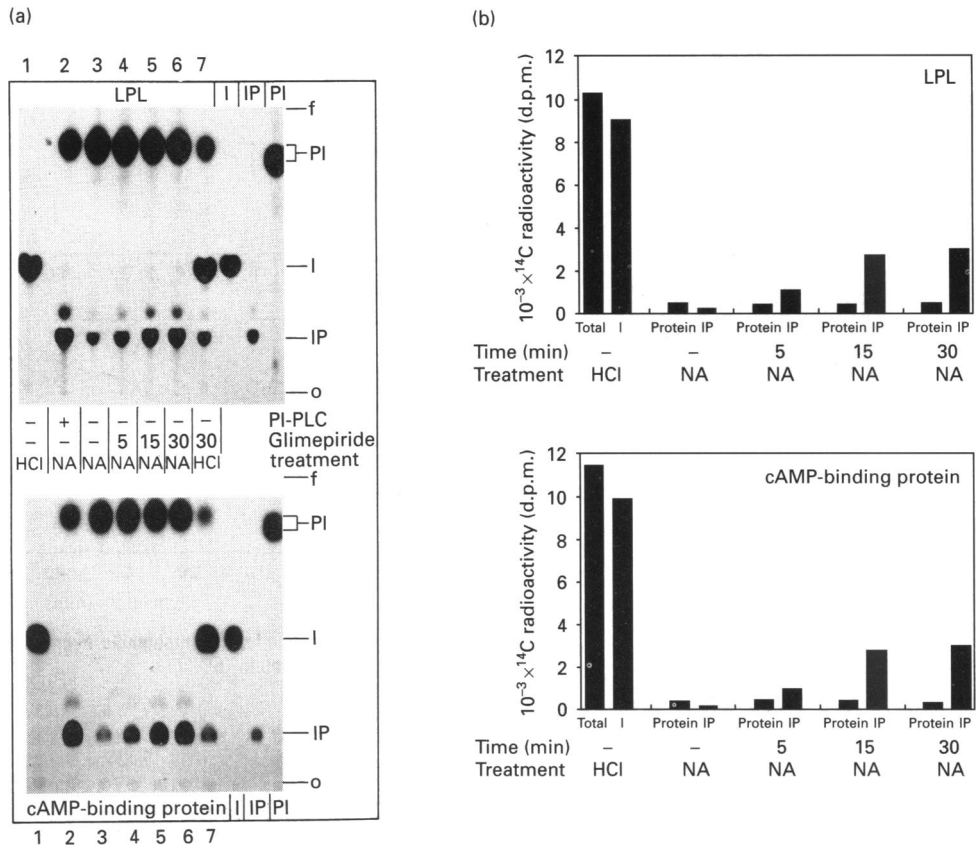

Figure 6 Identification of inositol as a component of cellular and released GPI-proteins

3T3 adipocytes were metabolically labelled with *myo*-[^{14}C]inositol and incubated in the absence or presence of 50 μM glimepiride for the times indicated or with 0.25 unit of PI-PLC (*Bacillus cereus*) per well for 2 h at 37 °C. The culture medium was removed and plasma membranes were prepared from the cells. Equivalent volumes of medium and amounts of membranes were immunoprecipitated with anti-LPL serum or affinity-purified by cAMP–Sepharose chromatography. The radiolabelled immunoprecipitated LPL or affinity-purified cAMP-binding protein was separated by SDS/PAGE, eluted from sliced gel pieces and dried under N_2. The plasma-membrane samples were resuspended in 0.5 ml of 4 M HCl and incubated at 110 °C for 5 h. The total HCl hydrolysates were applied directly on to a t.l.c. plate which was fluorographed (**a**, lane 1) and scanned for radioactivity (Berthold radioactivity scanner) (**b**). The radioactivity of the total hydrolysate applied (total) and of the spot co-migrating with the *myo*-inositol marker (**a**, I) are given (**b**, I). The medium samples were concentrated by evaporation (speedvac), subjected to nitrous acid deamination (NA) and subsequently precipitated with 5% trichloroacetic acid (4 °C, 1 h, then 10000 *g*, 5 min, two acetone washes). The pellet was dissolved in scintillation cocktail and counted for radioactivity (**b**, protein). The supernatant was neutralized, concentrated by freeze-drying and analysed by t.l.c. and fluorography (**a**, lanes 2–6). The radioactivity of the spot co-migrating with the *myo*-inositol phosphate marker (**a**, IP) was determined by liquid-scintillation counting of the corresponding spot material scraped from the plate (**b**, IP). As a control, an equivalent volume of the deaminated incubation mixture (30 min glimepiride; **a**, lane 6) was hydrolysed with 4 M HCl at 110 °C for 5 h after freeze-drying before t.l.c. analysis and fluorography (**a**, lane 7). ^{14}C-labelled *myo*-inositol (I), *myo*-inositol 1-phosphate (IP) and phosphatidylinositol (PI) were run in parallel as markers. Their positions and the origin (o) and front (f) are indicated on the right margin.

a steady state between synthesis/cell surface expression and release of the GPI-protein.

The correlation between the initial phase of GPI-protein activity loss in the plasma membrane and appearance in the culture medium strongly suggests release of the GPI-proteins from the plasma membrane in response to the sulphonylurea drug. To demonstrate this directly, cells were metabolically labelled with *myo*-[^{14}C]inositol or photoaffinity-labelled with 8-N_3-[^{32}P]cAMP and subsequently incubated for various times in the absence or presence of glimepiride with an excess of unlabelled *myo*-inositol or cAMP respectively. The medium and plasma membranes were analysed for membrane-bound and released radiolabelled proteins by their partitioning behaviour between a detergent (Triton X-114) and an aqueous phase. LPL (60 kDa) was identified by immunoprecipitation with a polyclonal antibody against human milk LPL (the specificity of the antibody has been established) whereas the cAMP-binding ectoprotein was visualized by photoaffinity labelling with 8-N_3-[^{32}P]cAMP as a single band (62 kDa) in the plasma-membrane fraction (G. Müller and W. Bandlow, unpublished work).

Figure 4 shows that, after 30 min of incubation in the absence of glimepiride, most of the LPL and cAMP-binding protein was localized at the plasma membrane (lane 1) and partitioned into the detergent phase (lane 2). Only a minor portion was detectable in the medium (lane 5) which may originate from broken cells. In contrast, in the presence of glimepiride the majority of both LPL and cAMP-binding protein was recovered from the medium (lane 10), most of which partitioned into the aqueous phase (lane 12). Intermediary levels of plasma-membrane-bound (lane 13), amphiphilic (lane 14) and soluble (lane 16), hydrophilic (lane 18) radiolabelled GPI-proteins were observed after a 5 min chase with glimepiride. The ratio, however, differed with the protein studied.

The kinetics of the decline in amphiphilic and increase in hydrophilic LPL and cAMP-binding protein were in close correlation (expressed as a ratio in the presence or absence of glimepiride) during 60 min of chase (see quantification in Figure 5). For up to 40 min the sum of the radiolabelled amphiphilic and hydrophilic versions remained nearly constant. Longer chase times markedly decreased the recovery, possibly as a result of

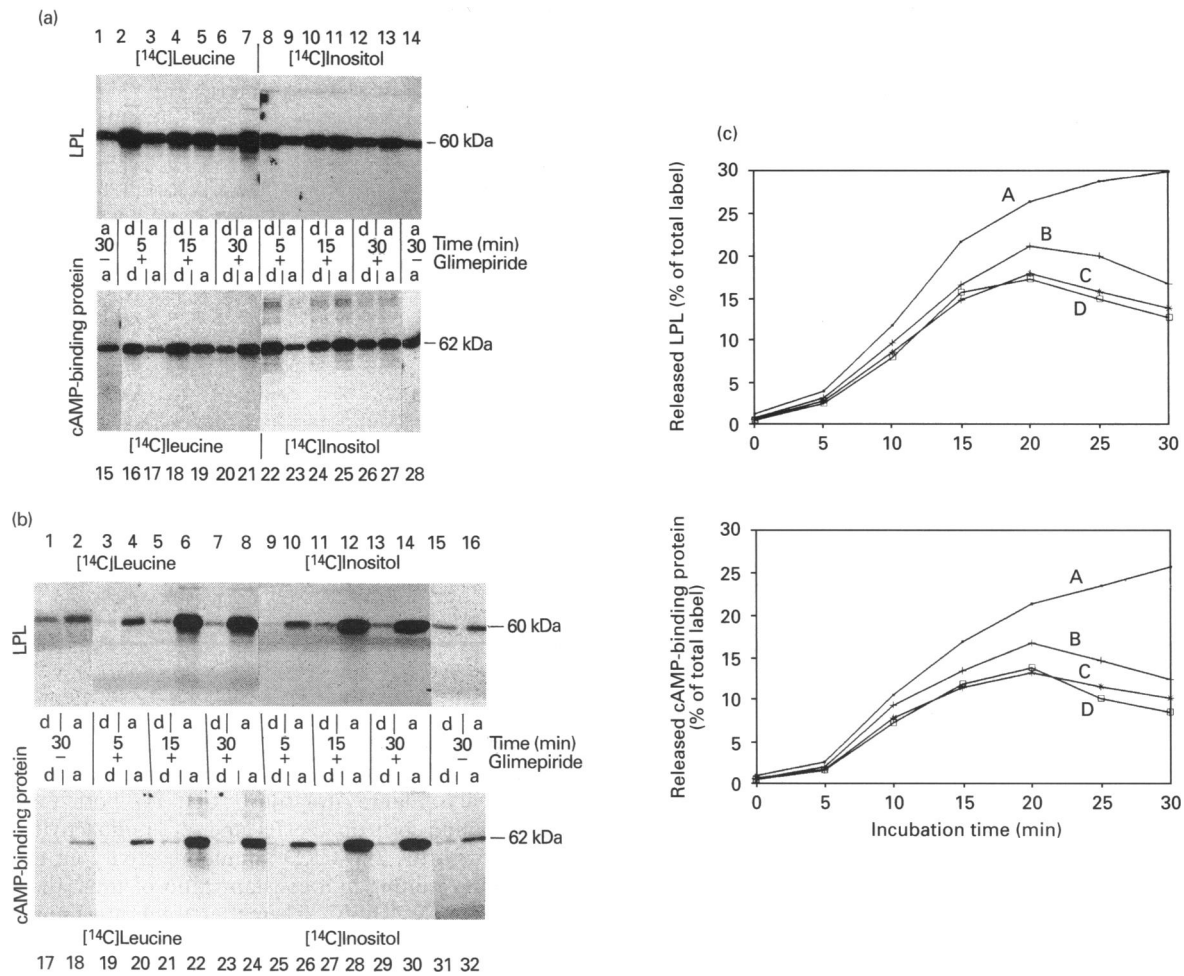

Figure 7 Retention of *myo*-inositol and anti-CRD immunoreactivity in GPI-proteins released from 3T3 adipocytes by glimepiride

(**a**) 3T3 adipocytes were metabolically labelled with *myo*-[^{14}C]inositol and [^{14}C]leucine and subsequently incubated in the absence or presence of 50 μM glimepiride for the times indicated. The culture medium was removed and plasma membranes were prepared from the cells. Equivalent volumes of the medium and membranes were subjected to Triton X-114 partitioning and subsequent immunoprecipitation with anti-LPL serum or affinity purification with cAMP–Sepharose chromatography. The immunoprecipitates (LPL, upper gels) or affinity-purified material (cAMP-binding protein, lower gels) of the detergent phase of the membrane samples (d) and of the aqueous phase of the medium samples (a) respectively were analysed by SDS/PAGE and fluorography. Similar d.p.m. values of [^{14}C]leucine- and *myo*-inositol-labelled protein were applied. The molecular masses were derived from metabolically labelled and immunoprecipitated LPL (60 kDa) and photoaffinity-labelled cAMP-binding protein (62 kDa) from adipocyte homogenate run in parallel. (**b**) The anti-LPL immunoprecipitates and affinity-purified material (amounts identical with **a**) were immunoprecipitated with anti-CRD antibodies and analysed by SDS/PAGE and fluorography. (**c**) 3T3 adipocytes were metabolically labelled with *myo*-[^{14}C]inositol (LPL, cAMP-binding protein; curves B and D) and [^{3}H]leucine (LPL; curves A and C), or alternatively were photoaffinity-labelled with 8-N_3-[^{3}H]cAMP (cAMP-binding protein; curves A and C), and subsequently incubated with 50 μM glimepiride for the times indicated. Samples of the culture medium were subjected to immunoprecipitation with anti-LPL serum (LPL) or trichloroacetic acid precipitation (cAMP-binding protein). One-half of the immunoprecipitates and trichloroacetate-precipitated material was analysed by SDS/PAGE, directly (curves A and B), the other half after immunoprecipitation with anti-CRD antibodies (curves C and D). The ^{3}H (curves A and C) and ^{14}C (curves B and D) radioactivities of total and anti-CRD immunoreactive LPL and cAMP-binding protein were measured with a Berthold scanner. The ^{3}H and ^{14}C radioactivities of total cellular immunoprecipitated LPL or affinity-purified cAMP-binding protein at time 0 were set at 100% each.

degradation of the soluble forms. The presence of cycloheximide did not affect the kinetics of the conversion up to 30 min of chase (results not shown). The amphiphilic/hydrophilic transition of the GPI-proteins can be best explained by proteolytic or lipolytic removal of the GPI moiety causing release of the protein from the outer face of the plasma membrane of 3T3 adipocytes into the medium.

The retention of radioactivity in the LPL and cAMP-binding ectoprotein from 3T3 adipocytes metabolically labelled with *myo*-[^{14}C]inositol for 14 h represents a first indication for a lipolytic cleavage event. Under these conditions most of the radioactivity of LPL and cAMP-binding protein was recovered in the inositol residue after total acid hydrolysis of the purified proteins, t.l.c. analysis and fluorography (Figure 6a, lane 1) or liquid-scintillation counting of the material co-migrating with the inositol marker (Figure 6b, HCl treatment). This is in agreement with the finding that the protein moiety of LPL and cAMP-binding ectoprotein prepared by nitrous acid deamination (which specifically cleaves between the inositol and non-acetylated glucosamine residues) instead of acid hydrolysis and subsequent trichloroacetic acid precipitation contained very little radioactivity (Figure 6b, protein).

We next studied whether the *myo*-[^{14}C]inositol moiety of LPL and cAMP-binding protein is retained quantitatively in the protein moiety after membrane release of the GPI-proteins by glimepiride. Cells were metabolically labelled in parallel with *myo*-[^{14}C]inositol or [^{14}C]leucine and incubated with glimepiride for various times. The GPI-proteins were purified from the plasma membrane and culture medium by Triton X-114 partitioning, immunoprecipitation (LPL) or affinity chromato-

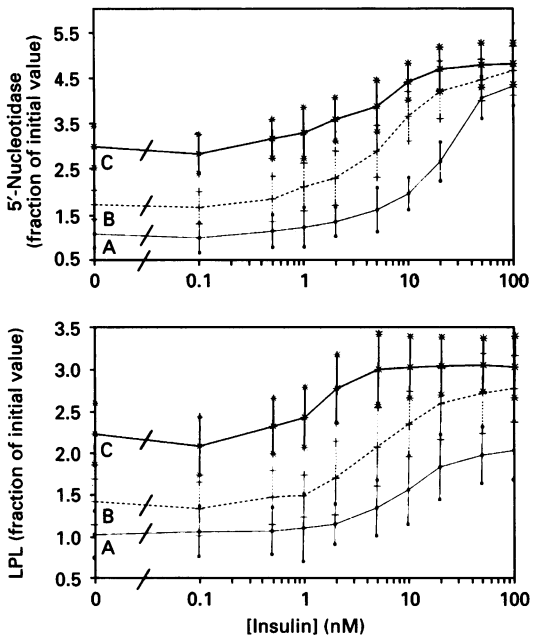

Figure 8 Effect of glimepiride on the insulin-stimulated release of GPI-proteins

3T3 adipocytes were incubated for 30 min in the absence (curve A) or presence of 5 μM (curve B) or 50 μM (curve C) glimepiride with various concentrations of insulin. 5'-Nucleotidase and LPL in the culture medium were assayed as described in the legend to Figure 1(a). Each point represents the means of three different experiments (cell preparations) with duplicate measurements for each.

Table 2 Release of GPI-proteins pretreated with cholera and pertussis toxins or proteinase inhibitors

3T3 adipocytes were pretreated as follows: (1) with cholera toxin (CT, 200 ng/ml) or pertussis toxin (PT, 100 ng/ml) for 20 h before addition of glimepiride, (2) with α_2-macroglobulin (MG, 1 mM), 4-(2-aminoethyl)benzylsulphonyl fluoride (ABF, 100 μM) or a mixture of leupeptin, pepstatin A and antipain dihydrochloride (each 10 μg/ml) for 5 min before addition of glimepiride, or (3) mock-treated for 20 h before glimepiride stimulation, and then incubated in the absence or presence of glimepiride (Gli; 50 μM) for 30 min. LPL and cAMP-binding protein in the culture medium and plasma membrane were assayed as described in legend to Figure 1(a). The values represent the means ± S.D. of three different experiments (cell preparations and pretreatments), each with triplicate determinations.

	LPL		cAMP-binding protein	
Treatment	Medium	Plasma membrane	Medium	Plasma membrane
–	1	1	1	1
Gli	2.22 ± 0.35	0.53 ± 0.12	4.38 ± 0.66	0.36 ± 0.08
PT + Gli	2.01 ± 0.28	0.60 ± 0.17	4.71 ± 0.81	0.25 ± 0.06
CT + Gli	2.43 ± 0.50	0.41 ± 0.09	4.55 ± 0.53	0.44 ± 0.09
MG + Gli	2.68 ± 0.43	0.38 ± 0.11	4.24 ± 0.70	0.31 ± 0.10
PF + Gli	2.11 ± 0.31	0.51 ± 0.17	4.02 ± 0.86	0.45 ± 0.14
Mix + Gli	2.07 ± 0.28	0.45 ± 0.10	3.97 ± 0.49	0.51 ± 0.12

graphy (cAMP-binding protein) and SDS/PAGE. The fluorogram (Figure 7a) shows that the increase in the amount of the [^{14}C]leucine-labelled hydrophilic form of both GPI-proteins in the medium throughout 30 min of incubation (lanes 3, 5, 7, 17, 19 and 21) was paralleled by a decrease in the amphiphilic form in the plasma membrane (lanes 2, 4, 6, 16, 18 and 20). The decrease was also observed for the [^{14}C]inositol-labelled GPI-proteins (lanes 8, 10, 12, 22, 24 and 26). In contrast, the kinetics of appearance of their hydrophilic versions reached a plateau after 15 min incubation with glimepiride (lanes 11, 13, 25 and 27), indicating the emergence of a portion of released molecules lacking inositol. The presence of a terminal inositol residue was confirmed by immunoprecipitation of a portion of the same material with anti-CRD antibodies (Figure 7b). The time course of immunoreactivity of the hydrophilic LPL and cAMP-binding protein correlated well with the *myo*-inositol (lanes 10, 12, 14, 26, 28 and 30) but not with the leucine label (lanes 4, 6, 8, 20, 22 and 24). The amphiphilic versions did not react with the anti-CRD antibodies to a significant extent demonstrating their high specificity. Only small amounts of hydrophilic total (Figure 7a, lanes 1, 14, 15 and 28) and immunoreactive (Figure 1b, lanes 2, 16, 18 and 32) GPI-proteins were observed after incubation for 30 min in the absence of glimepiride.

For quantification of the portion of released GPI-proteins without inositol, double labelling experiments with *myo*-[^{14}C]inositol and [^{3}H]leucine (LPL) or *myo*-[^{14}C]inositol and 8-N_3-[^{3}H]cAMP (cAMP-binding protein) were performed. After metabolic labelling, photoaffinity labelling and incubation of 3T3 adipocytes with glimepiride, LPL and the cAMP-binding ectoprotein recovered from the culture medium were immunoprecipitated and affinity-purified respectively, and analysed by SDS/PAGE directly or after immunoprecipitation with anti-CRD antibodies. The gels were scanned for ^{3}H/^{14}C radioactivity. Figure 7(c) shows that for both the LPL and cAMP-binding protein the ^{3}H and ^{14}C radioactivity increased together with the anti-CRD immunoreactivity up to 15 min of incubation resulting in a constant ratio of total and anti-CRD immunoreactive ^{3}H/^{14}C. Thereafter total and immunoreactive ^{14}C radioactivity remained constant with a decrease in the absolute values, whereas the amount of released total, but not immunoreactive ^{3}H-labelled LPL and cAMP-binding protein, increased further. Taken together, the identification of soluble GPI-proteins containing *myo*-inositol and exhibiting anti-CRD immunoreactivity strongly suggests that glimepiride causes rapid release of some GPI-proteins by lipolytic cleavage of their GPI membrane anchor. With longer incubation times, a portion of the released GPI-proteins lacks terminal *myo*-inositol as a result of hydrolytic cleavage(s) either within the glycosidic, phosphodiester or amide bonds of the GPI structure or proteolysis at the C-terminus. If the latter is the case, the proteinase involved should not be affected by a variety of proteinase inhibitors. No effect of α_2-macroglobulin, 4-(2-aminoethyl)benzylsulphonyl fluoride, leupeptin, pepstatin A or antipain dihydrochloride on glimepiride-induced release of LPL and cAMP-binding ectoprotein was detected (Table 2).

To identify the cleavage site of the putative phospholipase that acts during the initial phase of the releasing process, *myo*-[^{14}C]inositol-labelled LPL and cAMP-binding protein were isolated from the culture medium of 3T3 adipocytes after incubation with glimepiride and then subjected to nitrous acid deamination (Figure 6). After precipitation of the released protein moiety with trichloroacetic acid, the supernatant was analysed by t.l.c., fluorography (Figure 6a) and liquid-scintillation counting of the radiolabelled spot material (Figure 6b). The amount of the spot co-migrating with the inositol 1-phosphate marker (Figure 6a, IP) increased with the incubation time (Figure 6a, lanes 3–6; Figure 6b, IP). The nature of this material (30 min incubation) was confirmed by total acid hydrolysis which almost completely converted inositol phosphate into inositol (Figure 6a, lane 7). The marginal amount of radiolabelled inositol detected

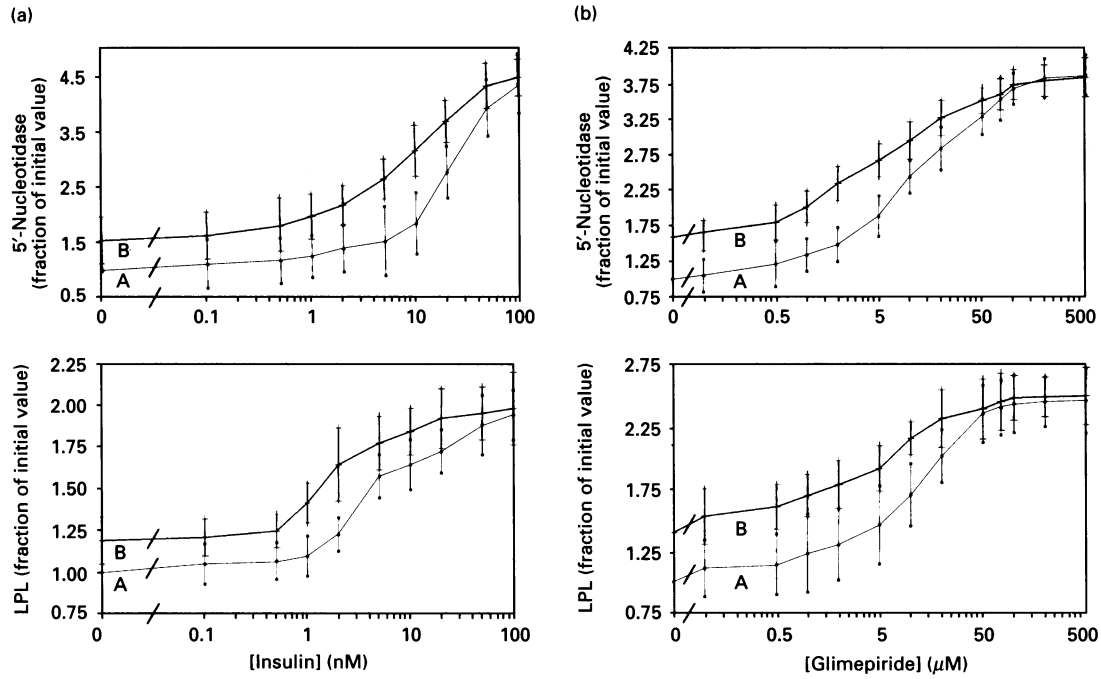

Figure 9 Effect of long-term treatment with glimepiride on release of GPI-proteins

3T3 adipocytes were preincubated in the absence (curve A) or presence (curve B) of glimepiride (50 μM) for 16 h. Subsequently the cells were washed three times with DMEM and incubated in the absence or presence of various concentrations of insulin (**a**) or glimepiride (**b**) for 30 min. 5′-Nucleotidase and LPL in the culture medium were assayed as described in the legend to Figure 1(a).

after incubation with glimepiride may be due to phosphatase action in the culture medium or during isolation of the proteins. The identification of inositol phosphate in the released GPI-proteins, as was observed after a control incubation of 3T3 adipocytes with bacterial PI-PLC (Figure 6a, lane 2), indicates the activation of a GPI-PLC by glimepiride in 3T3 adipocytes. The generation of radiolabelled phosphatidylinositol after incomplete digestion of the GPI-proteins by PI-PLC and glimepiride (Figure 6a, lanes 2–6) again confirmed the metabolic incorporation of *myo*-[^{14}C]inositol.

Since some bacterial toxins are known to interfere with hormone-stimulated PLC action, we investigated the effect of *Bordetella pertussis* and *Vibrio cholerae* toxins on glimepiride-induced release of the GPI-proteins. Pretreatment of 3T3 adipocytes with both toxins did not affect basal or drug-induced specific activities of the LPL or cAMP-binding protein in the plasma membranes or culture medium (Table 2). Under the same conditions, pertussis toxin blocked the early insulin-stimulated generation of dimyristoylglycerol (results not shown). Thus the release of these GPI-proteins does not seem to be catalysed by PLC which is regulated via pertussis or cholera toxin-sensitive G-proteins.

In addition to sulphonylurea agents, shown in this study, insulin has been reported to stimulate release of several GPI-proteins from 3T3-L1 adipocytes by degradation of their GPI membrane anchor (see the Discussion). Therefore we were interested in the relationship between the drug- and insulin-induced membrane-releasing mechanisms. Cells were incubated with either 5 or 50 μM glimepiride in combination with increasing concentrations of insulin (0.1–100 nM) before the medium was assayed for LPL and 5′-nucleotidase (Figure 8). Glimepiride at both concentrations caused a significant increase in enzyme activity in the medium compared with insulin stimulation alone (Figure 8, curve A). At 5 μM glimepiride and 1–20 nM insulin, the total release (Figure 8, curve B) exceeded the sum of the insulin and glimepiride effects alone, indicative of synergistic effects of glimepiride and insulin. At 50 μM glimepiride and insulin below 10 nM, the effects of glimepiride and insulin were additive, and, with insulin above 10 nM, they were non-additive. The synergistic release at low and the non-additive release at high concentrations of glimepiride and insulin represent a first clue that the sulphonylurea drug and insulin use at least one common step for membrane release of GPI-proteins.

Chronic treatment with sulphonylurea drugs has been reported to increase insulin sensitivity *in vivo* and *in vitro* (see the Introduction). Therefore we investigated whether long-term incubation of 3T3 adipocytes with glimepiride enhances the insulin-stimulated release of LPL and 5′-nucleotidase (Figure 9). To this end, 3T3 adipocytes were incubated with glimepiride (50 μM) for 16 h, then washed extensively to remove the drug and already released GPI-proteins and subsequently treated with various concentrations of insulin (Figure 9a) for 30 min after which the specific activities of LPL and 5′-nucleotidase in the culture medium were assayed. Long-term incubation with glimepiride (Figure 9a, curve B) resulted in a significant leftward-shift of the dose–response curve for subsequent acute stimulation of membrane release of the GPI-proteins by insulin. The maximal rate of release was not increased significantly. Analogously, long-term treatment with glimepiride increased the sensitivity of the GPI-protein release for glimepiride (Figure 9b). Interestingly, pretreatment with glimepiride caused a marked release of LPL and 5′-nucleotidase during the subsequent 30 min of incubation in the absence of insulin (Figure 9a) or glimepiride (Figure 9b). Thus either glimepiride was not removed completely before the

short-term treatment or the releasing mechanism, once activated by the drug, persists for a prolonged period of time.

DISCUSSION

This paper demonstrates that the novel sulphonylurea drug, glimepiride, stimulates the release of three ectoproteins, LPL, 5′-nucleotidase and a 62 kDa cAMP-binding protein, from the plasma membrane of 3T3 adipocytes into the culture medium. This is based on the time- and concentration-dependent increase in the enzyme activity of these proteins in the medium accompanied by a parallel decrease in enzyme activity in the plasma membrane. Furthermore, radiolabelled LPL and cAMP-binding protein are converted from the membrane-bound amphiphilic form into the soluble hydrophilic form during incubation with glimepiride.

It is possible that the sulphonylurea- and insulin-dependent releasing mechanisms share some common steps, since at submaximal concentrations the two agents act in a synergistic manner and at higher concentrations they do not exceed a maximal level of release. Up until now, insulin-regulated membrane release of GPI-proteins has been reported for 3T3-L1 adipocytes [LPL (Eckel et al., 1978; Spooner et al., 1979; Chan et al., 1988); several unidentified proteins (Lisanti et al., 1989); 5′-nucleotidase and cAMP-binding ectoprotein (this study)], BC_3H1 myocytes [alkaline phosphatase (Romero et al., 1988)] and HepG-2 cells [heparan sulphate proteoglycan (Ishihara et al., 1987)], for membranes prepared from rat hindquarter muscles after perifusion with insulin [5′-nucleotidase (Klip et al., 1988)] and for rat osteosarcoma cells after culture in the presence of insulin [alkaline phosphatase (Levy et al., 1984)].

The retention of *myo*-inositol phosphate and appearance of anti-CRD cross-reactivity in the soluble GPI-proteins during the initial phase of glimepiride-induced release indicate cleavage of the GPI membrane anchor by GPI-PLC. Degradation of the PI anchor has also been suggested for the insulin-stimulated release of LPL (Chan et al., 1988). However, the appearance of released (and enzymically active) LPL and cAMP-binding ectoprotein without *myo*-inositol and anti-CRD immunoreactivity during later stages of incubation hints at additional hydrolytic processing near the GPI attachment site which does not decrease the apparent size of the proteins in SDS/PAGE. These molecules may originate from either membrane-anchored or lipolytically released GPI-proteins. The kinetics of their appearance after a large pool of lipolytically released proteins has accumulated suggests the latter possibility, i.e. two sequential distinct lipolytic and hydrolytic processing events.

The mechanism by which sulphonylurea drugs stimulate the GPI-PLC remains to be elucidated. A sulphonylurea receptor has been described in pancreatic β-cells and the brain (Kramer et al., 1988) and characterized as a putative component of an ATP-dependent K^+ channel [for a review see Boyd (1988)] which, however, is absent from 3T3-L1 adipocytes and BC_3H1 and L6 myocytes (Rajan and Boyd, 1988; Cooper et al., 1990; Davidson et al., 1991). Binding studies indicate the existence also of specific sulphonylurea receptors in isolated rat adipocytes (Martz et al., 1989). They have not been characterized so far, but may form a component of the signal-transduction cascade through which sulphonylurea drugs activate a GPI-PLC in extrapancreatic insulin-sensitive cells. It is unknown whether this PLC is structurally related to the insulin-stimulated GPI-PLC from rat liver membranes (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986), the only GPI-PLC in mammalian cells described so far.

The functional significance of the regulated release of GPI-ectoproteins from the plasma membrane remains a matter of speculation. It may represent a means to down-regulate the cell surface expression of these proteins with respect to their turnover or reduced requirement during some physiological situation. Alternatively, the activity of the GPI-proteins released into the extracellular space *per se* may be of physiological importance. The functional state and role of solubilized GPI-proteins in extracellular fluids is not completely understood [for a review see Low (1987) and Low and Saltiel (1988)]. LPL released from parenchymal cells of various tissues moves through the vascular endothelium and binds to the capillary luminal surface where it hydrolyses di- and tri-acylglycerols. The insulin-deficient state (during uncontrolled type I diabetes) is accompanied by low plasma LPL activity and hypertriglyceridaemia (Bagdade et al., 1967), pointing to a special need for soluble LPL. Although the function of the cAMP-binding ectoproteins, recently identified in lower and higher eukaryotes, is not known, it may be of interest in this respect that the yeast GPI-anchored 54 kDa cAMP-binding ectoprotein, which is structurally related to the cAMP-binding ectoprotein of 3T3 adipocytes, exhibits different cAMP-binding characteristics and an additional amino acid photoaffinity-labelled with 8-N_3-[^{32}P]cAMP after cleavage of its GPI membrane anchor with exogenous GPI-PLC from *Trypanosoma brucei* (G. Müller and W. Bandlow, unpublished work). This hints at the possibility that the removal of the GPI modification and/or the altered localization may cause changes in the conformation and function of GPI-proteins. A physiological function may also reside in the potential cleavage products of PLC action on the GPI moiety and limited proteolysis at the C-terminus of the GPI-proteins, diacylglycerol and GPI-peptides or phosphoinositolglycan peptides. These molecules may function as chemical mediators for some of the stimulatory effects of sulphonylurea on glucose utilization in isolated rat adipocytes and cultured 3T3-L1 adipocytes (Jacobs et al., 1989; Farese et al., 1991).

Our data imply that the novel sulphonylurea compound, glimepiride, is not only valuable as an oral antidiabetic drug (Geisen, 1988), but may be useful for elucidation of the mode of action of this class of therapeutic agents in extrapancreatic tissues.

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