BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 749–770

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

# Induced translocation of glycosylphosphatidylinositol-anchored proteins from lipid droplets to adiposomes in rat adipocytes

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**Background and purpose:** Adipocytes release membrane vesicles called adiposomes, which harbor the glycosylphosphatidylinositol-anchored proteins (GPI proteins), Gce1 and CD73, after induction with palmitate, $H_2O_2$ and the sulphonylurea drug glimepiride. The role of lipid droplets (LD) in trafficking of GPI proteins from detergent-insoluble, glycolipid-enriched, plasma membrane microdomains (DIGs) to adiposomes in rat adipocytes was studied.
**Experimental approach:** Redistribution of Gce1 and CD73 was followed by pulse-chase and long-term labelling, western blot analysis and activity determinations with subcellular fractions and cell-free systems exposed to palmitate, $H_2O_2$ and glimepiride.
**Key results:** In response to these signals, Gce1 and CD73 disappeared from DIGs, then transiently appeared in LD and finally were released into adiposomes from small, and, more efficiently, large adipocytes. From DIGs to LD, Gce1 and CD73 were accompanied by cholesterol. Cholesterol depletion from DIGs or LD caused accumulation at DIGs or accelerated loss from LD and release into adiposomes, respectively, of the GPI proteins. Blockade of translocation of Gce1, CD73, caveolin-1 and perilipin-A from DIGs to LD blocked LD biogenesis and long term-accumulation of LD interfered with induced release of the GPI proteins into adiposomes. GPI protein release was up-regulated upon long term-depletion of LD. Adiposomes were released by a DIGs-based cell-free system, but only in presence of LD.
**Conclusions:** GPI proteins are translocated from DIGs to LD prior to their release into adiposomes, which is regulated by cholesterol, LD content and LD biogenesis. This detour may serve to transfer information about the LD content and to control lipolysis/esterification between large and small adipocytes *via* GPI protein-harbouring adiposomes.
*British Journal of Pharmacology* (2009) **158,** 749–770; doi:10.1111/j.1476-5381.2009.00360.x; published online 24 August 2009

**Keywords:** glycosylphosphatidylinositol; lipid droplets; membrane microdomains; microvesicles; exosomes; adipocytes; sulphonylurea drugs; lipid metabolism

**Abbreviations:** $AACOCF_3$, arachidonyl trifluoromethylketone; AMPCP, α,β-methlyene-ADP; BEL, bromoenol lactone; BFA, brefeldin-A; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; $cPLA_2\alpha$, cytosolic group IVA phospholipase $A_2\alpha$; cPLAI, $cPLA_2$-specific inhibitor delivered by Calbiochem-Merck; DIGs, detergent-insoluble glycolipid-enriched plasma membrane microdomains; DPI, diphenylene iodonium; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; 5′-FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine; GO, glucose oxidase; (GPI-)PLC, (glycosylphosphatidylinositol-specific) phospholipase C; GPI proteins, glycosylphosphatidylinositol-anchored proteins; $iPLA_2$, group VI phospholipase $A_2$; LD, lipid droplets; MAFP, methylarachidonyl fluorophosphate; β-mCD, β-methyl-cyclodextrin; MFG-E8, milk fat globule membrane epidermal growth factor 8; NADPH, β-nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PC, phosphatidylcholine; PY-2, pyrrolidine-2; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TAG, triacylglycerol

## Introduction

During the past decade, mammalian adipocytes have been recognized as secretory cells for a multitude of soluble hormones, such as the cytokines leptin and interleukin-6, which act as autocrine, paracrine or endocrine messengers for information about the metabolic (lipid-laden) state of the adipocytes (Bradley *et al.*, 2001; Kershaw and Flier, 2004). The release of membrane-associated enzymes, such as Src kinase, and structural proteins, such as caveolin-1, incorporated into microvesicles and exosomes from cultured 3T3-L1 adipocytes (Aoki *et al.*, 2007) and primary rat adipocytes (Aoki *et al.*, 2007; Müller *et al.*, 2009), has only recently been reported. These small membrane vesicles differ in size (0.1–1 μm vs.

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Received 6 January 2009; revised 6 March 2009; accepted 30 March 2009

30–100 nm) and in the apparent buoyant density during sucrose gradient centrifugation (1.25–1.30 vs. 1.13–1.21 g·mL$^{-1}$), reflecting partly distinct but overlapping phospholipid and protein compositions of their bilayers. Interestingly, the release of these so-called adiposomes (Aoki *et al.*, 2007) from adipocytes was found to be strongly up-regulated by certain physiological (high concentrations of palmitate, glucose or $H_2O_2$) or pharmacological signals (antidiabetic sulphonylurea, glimepiride) (Müller *et al.*, 2009). However, as yet, there are no data concerning the physiological function of released adiposomes within adipose tissue or in other tissues and organs, although microvesicles and exosomes derived from various blood- and endothelium-derived cells have been shown to exert physiological effects on other tissues and organs (Wolf, 1967; Poste and Nicolson, 1980; Heijnen *et al.*, 1999; Stoorvogel *et al.*, 2002; Thery *et al.*, 2002; Freyssinet, 2003; Fevrier and Raposo, 2004; Al-Nedawi *et al.*, 2008).

Transmembrane (caveolin-1), peripheral membrane (Src kinases) and soluble cytosolic (heat shock protein 70) proteins are constituent components of adiposomes (Aoki *et al.*, 2007; Müller *et al.*, 2009), and members of the class of glycosylphosphatidylinositol (GPI)-anchored proteins (GPI proteins) have been found associated with adiposomes from rat adipocytes upon challenge with high concentrations of palmitate, $H_2O_2$ and glimepiride (Müller *et al.*, 2009).

GPI proteins are typically embedded in the detergent-insoluble, glycolipid-enriched, membrane microdomains (DIGs) of the outer leaflet of the plasma membrane *via* their covalently coupled glycolipid anchor, with their protein moiety facing the cell surface. (Ikezawa, 2002). DIGs [which probably serve for the functional compartmentalization of membranes (Brown and London, 1998; Varma and Mayor, 1998)] are rich in cholesterol, (glyco)sphingolipids, acylated proteins such as tyrosine kinases of the Src-family, certain transmembrane proteins, such as the cholesterol-binding transmembrane and structural coat protein caveolin (Öst *et al.*, 2005), and GPI proteins (Müller and Frick, 1999). They can be isolated on sucrose density gradients owing to their insolubility in non-ionic detergents at low temperature and low buoyant density. In the course of expression of caveolin-1 in terminally differentiated cells, such as adipocytes, DIGs form small flask-shaped invaginations, so-called caveolae, which undergo endocytosis (Anderson, 1993).

Intriguingly, the GPI proteins, Gce1 and CD73 – which are apparently involved in the degradation of cyclic adenosine monophosphate (cAMP) on the basis of their cAMP-specific phosphodiesterase and 5′-nucleotidase activities, respectively (Müller *et al.*, 2008c) – are also recovered with lipid droplets (LD) prepared from palmitate-, $H_2O_2$- and glimepiride-induced adipocytes (Müller *et al.*, 2008b). LD are intracellular organelles specialized for the storage of triacylglycerol (TAG) and cholesteryl ester in their core. LD are surrounded by a monolayer of phospholipids and free cholesterol with embedded LD-specific PAT proteins, such as perilipin-A (Ducharme and Bickel, 2008; Fujimoto *et al.*, 2008). In addition, typical resident proteins of DIGs, such as caveolin, have recently been identified in LD (Pol *et al.*, 2005; Robenek *et al.*, 2005). Originally, caveolin was believed to be mis-sorted to the LD as only over-expressed, mutant or truncated versions were observed on the LD (Martin and Pol, 2005). However, the presence of wildtype endogenous caveolin has been demonstrated on newly formed primordial LD of rat adipocytes (Marchesan *et al.*, 2003), as well as those of almost fully differentiated 3T3-L1 adipocytes (Blouin *et al.*, 2008). The function of caveolin on the LD is not fully understood, but a role of caveolin-rich plasma membrane DIGs in the biogenesis of peripheral LD has been proposed (Örtegren *et al.*, 2007).

Current working hypotheses suggest fundamentally different mechanisms for the biogenesis of LD (Martin and Parton, 2006; Robenek *et al.*, 2006; Ducharme and Bickel, 2008; Fujimoto *et al.*, 2008; Thiele and Spandl, 2008), DIGs (Brown and London, 1998; Varma and Mayor, 1998), exosomes (Johnstone *et al.*, 1987; Heijnen *et al.*, 1999; Stoorvogel *et al.*, 2002; Thery *et al.*, 2002) and microvesicles (Heijnen *et al.*, 1999; Freyssinet, 2003). Hence, it is difficult to explain the multiple expression of Gce1, CD73, caveolin-1 and perilipin-A in each of these subcellular structures (Müller *et al.*, 2009). LD are generally believed to form by inundation between the leaflets of the endoplasmic reticulum membrane, and, possibly, the plasma membrane, followed by the distension of the membrane and final budding of the cytoplasmic membrane leaflet (Martin and Parton, 2006; Ducharme and Bickel, 2008; Fujimoto *et al.*, 2008; Thiele and Spandl, 2008). PAT proteins may be transferred concomitantly to the LD surface or targeted subsequently from cytosolic sites of synthesis to the nascent LD. Furthermore, the endocytosis of caveolae has recently been suggested to mediate the cholesterol-induced trafficking of caveolin-1 to LD in differentiating adipocytes (Pol *et al.*, 2005; Le Lay *et al.*, 2006; Blouin *et al.*, 2008). Fusion of nascent small LD, progressive direct accretion of lipids and proteins from the endoplasmic reticulum, plasma membranes or cytosol onto the surface of nascent LD and non-vesicular extrusion of proteins from the endoplasmic reticulum membrane or plasma membrane (Öst *et al.*, 2005; Robenek *et al.*, 2005; 2006), may contribute to the apparent complexity and versatility of LD biogenesis.

The biogenesis of exosomes involves the segregation of proteins within the endosomal membranes, followed by their inward budding together with selected cargo into the endosomal lumen. The subsequent movement of the resulting intra-endosomal vesicles, the so-called multivesicular bodies, to and fusion with the plasma membrane, finally results in the release of the exosomes. Importantly, cholesterol, the gangliosides GM1 and D3, acylated protein kinases of the Src-family, the caveolin functional analogues flottilin-1 and stomatin, and the GPI proteins CD55, CD58 and CD59, are all considered to reside within DIGs and to be present in the human reticulocyte-, B cell- and dendritic cell-derived exosomes (Rabesandratana *et al.*, 1998; Thery *et al.*, 2001; Wubbolts *et al.*, 2003). These observations suggest that exosomes may be formed at DIGs, which are expressed in the endosomal membranes and contribute to molecule segregation and sorting into the multivesicular bodies, which has been seen in electron microscopic (De Gassert *et al.*, 2003) and proteomic (Kang *et al.*, 2008) analyses of various cell types. A biogenetic relationship between exosomes and DIGs/caveolae may also be deduced from the previous demonstration of intracellular trafficking of certain GPI proteins and caveolin-1 (in a cholesterol-dependent fashion) from DIGs *via* non-clathrin-

coated vesicles to endosomes (Keller *et al*., 1991; Mayor *et al*., 1998) from which exosomes may be formed.

Microvesicles are formed in mammalian cells by shedding of the plasma membrane due to cytosolic $Ca^{2+}$-induced transverse migration of phosphatidylserine resulting in a transient overload of the outer leaflet at the expense of the inner one (Trams *et al*., 1981; Salzer *et al*., 2002). It remains to be clarified whether DIGs can operate as preferential sites of shedding, which would explain the predominant sorting of some typical DIGs constituent proteins into microvesicles (Dolo *et al*., 2000; Del Conde *et al*., 2005).

The observed multiple localization of Gce1, CD73, perilipin-A and caveolin-1 at adiposomes, DIGs, and LD might be accomodated by the following biogenetic relationships: (i) LD and adiposomes may be released from special plasma membrane DIGs engaged in the simultaneous biogenesis of both peripheral LD and microvesicles from the cytoplasmic leaflet and membrane bilayer, respectively; (ii) LD and adiposomes may be released from endoplasmic reticulum/endosomal sites capable of the simultaneous biogenesis of both central LD and exosomes from the cytoplasmic leaflet and membrane bilayer, respectively; and (iii) Gce1, CD73, perilipin-A and caveolin-1 may successively move from plasma membrane DIGs *via* peripheral/central LD to special sites (DIGs ?) within the plasma membrane and endosomes for their final incorporation into microvesicles and exosomes respectively. The present study provides strong evidence that in rat adipocytes Gce1, CD73, caveolin-1 and perilipin-A are translocated consecutively along DIGs, LD and adiposomes in response to palmitate, glimepiride and $H_2O_2$ rather than being incorporated into LD and adiposomes in parallel at plasma membrane, endoplasmic reticulum or endosomal sites (DIGs ?). This multi-step itinerary suggests a biogenetic and functional coupling between proteins located at the cell surface, LD and adiposomes.

## Methods

*Animals*

All animal care and procedures were in accordance with the German Animal Protection Law. Rats (see below) were obtained from Charles-River Laboratories (Wilmington, MA, USA).

*Preparation and incubation of rat isolated adipocytes*

Adipocytes isolated by collagenase digestion from epididymal fat pads of male Sprague Dawley rats (140–160 g, fed *ad libitum*) under sterile conditions according to published procedures (Müller *et al*., 1997) were suspended in 1 mL of adipocyte buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 20 mM HEPES/KOH, pH 7.4), supplemented with 0.2% (w/v) bovine serum albumin (BSA), 100 $\mu g \cdot mL^{-1}$ gentamycin, 1 mM sodium pyruvate and 5.5 mM D-glucose at $0.7 \times 10^6$ cells per $mL^{-1}$ in a shaking water bath (100 cycles$\cdot min^{-1}$, 37°C) under constant bubbling, with 5% $CO_2$/95% $O_2$ for incubation with the agents indicated. The preparation of stock solutions of insulin, palmitate and glimepiride coupled to BSA was outlined previously (Müller *et al*., 2008d). Recombinant glucose oxidase (GO) in 10 mM phosphate buffer (pH 7.4) at 10 $U \cdot mL^{-1}$ was diluted in adipocyte buffer immediately before use.

*Separation of large and small adipocytes*

Adipocytes prepared from 3-month old male rats by collagenase digestion were filtered through serial nylon mesh screens with pore sizes of 75, 150 and 400 μm to obtain small (diameter < 75 μm) and large (diameter > 400 μm) adipocytes. Cell number was determined after fixation of aliquots of the adipocytes with osmic acid using a Coulter counter. Total lipid content of the adipocyte suspension ('lipocrit') was measured as described previously (Müller and Wied, 1993). Mean adipocyte size was determined as the mean cellular lipid content (mL per cell) by dividing of the total lipid content of the cell suspension (mL) by the cell number $mL^{-1}$, as described previously (Cushman and Salans, 1978).

*Primary culture of rat adipocytes*

Isolated rat adipocytes ($1 \times 10^5$ cells$\cdot mL^{-1}$) were incubated (20 h, 37°C) in 10 mL of adipocyte buffer supplemented with 5.5 mM glucose, 4 mM sodium pyruvate, 16 mM glutamine, 2% (w/v) BSA, 100 $\mu g \cdot mL^{-1}$ gentamycin, 50 units$\cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ streptomycin sulphate, as described previously (Müller and Wied, 1993) in 50 mL polystyrene tubes in the absence or presence of isoproterenol and orlistat as indicated. Subsequently, the cells were washed three times by flotation with 50 mL each of adipocyte buffer, suspended in 2.5 mL of adipocyte buffer (final titer $0.4 \times 10^5$ cells$\cdot mL^{-1}$) and incubated as indicated. The differently incubated adipocytes were subjected to the same washing and flotation procedures to compensate for variations in cell titer and viability.

*Metabolic labelling of rat adipocytes with [$^{14}$C]inositol*

Untreated rat adipocytes (5 mL, $2 \times 10^6$ cells$\cdot mL^{-1}$) were washed twice with 50 mL of labelling medium (adipocyte buffer supplemented with 4 mM sodium pyruvate and 0.5% BSA) and then incubated (2 h, 37°C) in 20 mL of labelling medium supplemented with 50 units$\cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ streptomycin sulfate in 150 mL culture flasks under 95% $O_2$/5% $CO_2$. Labelling was started by the addition of *myo*-[U-$^{14}$C]inositol (20 μCi, 0.1 mM final conc.). After incubation (10 min for pulse or 5 h for long term labelling; 37°C), the agents were added, and the incubation continued as indicated. Thereafter, the labelling was terminated by the addition of 2 mL of labelling medium supplemented with 50 mM glucose and 10 mM *myo*-inositol.

*Metabolic labelling of rat adipocytes with [$^{14}$C]acetate*

Rat adipocytes ($1 \times 10^6$ cells) were incubated (5 min for pulse or 1 h for long term labelling, 37°C) in primary culture in 1 mL of adipocyte buffer supplemented with 0.2% (w/v) BSA, 100 $\mu g \cdot mL^{-1}$ gentamycin and [1-$^{14}$C]acetate (100 μM, 0.5 μCi, 50 $\mu Ci \cdot \mu mol^{-1}$) as described previously (Keay and Grossberg, 1980). Thereafter, the adipocytes were separated from the medium by flotation, washed once with adipocyte buffer and

then incubated (37°C) with adipocyte buffer supplemented with 10 mM acetate and 4 mM sodium pyruvate. After various periods of time, 10 mL of toluene-based scintillation cocktail was added. After phase separation (20 h, 25°C), the upper organic phase was removed and measured for radioactivity by liquid scintillation counting.

*Determination of radiolabelled cholesterol*

Adipocytes metabolically labelled with [$^{14}$C]acetate were separated from the incubation medium by flotation, washed three times with adipocyte buffer supplemented with 2% (w/v) BSA and then extracted for lipids using chloroform/methanol (2/1, v/v). Major lipid classes were separated by thin layer chromatography (hexane/diethyl ether/acetic acid, 70/30/1, v/v) and identified after rhodamine dye fluorimetry by comparing migration of purified (>99%) standard lipids (TAG, cholesteryl ester, cholesterol, fatty acids) run in parallel. Areas of the thin-layer gel containing peak fluorescence and radioactivity for cholesterol were scraped into 10 mL of toluene-based scintillation cocktail and counted for radioactivity. All values were corrected for control incubations lacking cells. Control experiments demonstrated that under these conditions with basal adipocytes 75–80% of acetate is incorporated into free cholesterol, 15–18% into TAG and less than 2% into free fatty acids. Induction of the adipocytes with GO, glimepiride or palmitate did not significantly affect this distribution.

*Preparation and purification of adiposomes*

After incubation of the adipocytes, 250 µL portions of the total incubation medium were transferred (continuous shaking) into microfuge tubes (Beckman) pre-filled with 100 µL of dinonylphthalate and then centrifuged (1200× *g*, 1 min, 20°C). The tubes were cut through the dinonylphthalate layer separating the adipocytes at the top from the incubation medium in the lower part of the tubes, which was retained. Care was taken to minimize the volume of dinonylphthalate taken along with the incubation medium into the lower part. After transfer of the incubation medium into 1 mL Eppendorf cups and washing the lower part of the tube once with 250 µL of adipocyte buffer containing 0.5 mM dithiothreitol (DTT) and protease inhibitor mix 'complete' (1 tablet per 100 mL), the combined medium and washing fluid were centrifuged (3000× *g*, 20 min, 4°C) for the removal of cell debris. One hundred microlitre portions of the supernatant obtained were centrifuged (Beckman Airfuge, Kregfeld, Germany, A-110 fixed angle rotor, 160 000× *g*, 30 min, 4°C). After careful aspiration of the supernatants, the pellets were suspended in 20 µL of adiposome buffer (10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 140 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM isobutylmethylxanthine, 1 mM DTT, 20 mM sodium fluoride, 25 mM glycerol-3-phosphate, 10 mM sodium pyrophosphate and protease inhibitor mix as described above), combined and re-centrifuged as above. The pellet was washed (vortexing) two times with 100 µL each of adiposome buffer.

*Photoaffinity-labelling of adipocytes and adiposomes*

Adipocytes collected from 1 mL incubation mixtures ($7 \times 10^5$ cells) by flotation (500× *g*, 1 min, 20°C) and aspiration of the infranatant were diluted with adipocyte buffer (75 µL final vol.) and then incubated (15 min, 15°C) with 5 Ci 8-$N_3$-[$^{32}$P]cAMP (100 pmol) in the presence of 1 mM AMP for labelling of Gce1 or 15 µCi [$^{14}$C]5′-*p*-fluorosulphonylbenzoyladenosine (5′-FSBA, 20 nmol) in the presence of 100 µM ADP for labelling of CD73 (90 µL final vol.) in the wells of microtiter plates (96 well) under mild shaking followed by irradiation with UV light (254 nm, 8000 µW·cm$^{-2}$) at a distance of 0.5 cm for 1 min (Müller *et al*., 2008a) and d). The reactions were quenched by addition of 10 µL of the same buffer containing 20 mM cAMP (in case of 8-$N_3$-[$^{32}$P]cAMP) or AMP (in case of [$^{14}$C]5′-FSBA).

Adiposomes collected from 2.5-mL incubation mixtures ($17.5 \times 10^5$ adipocytes) were suspended in 40 µL of adiposome buffer. After incubation (15 min, 4°C) with 0.1 µCi 8-$N_3$-[$^{32}$P]cAMP (2 pmol) in the presence of 1 mM AMP for labelling of Gce1 or 0.75 µCi [$^{14}$C]5′-FSBA (1 nmol) in the presence of 100 µM ADP for labelling of CD73 (50 µL final vol.) in the wells of microtiter plates (96 well), the mixtures were irradiated with UV light (254 nm, 8000 µW·cm$^{-2}$) at a distance of 0.5 cm for 1 min (Müller *et al*., 2008a and d). The reactions were quenched by addition of 50 µL of the same buffer containing 10 mM cAMP/AMP for labelling with 8-$N_3$-[$^{32}$P]cAMP/[$^{14}$C]5′-FSBA.

*Cell-free system for the release of adiposomes*

DIGs were prepared from GO-stimulated rat adipocytes ($1.75 \times 10^5$ cells), which had been photoaffinity labelled with 8-$N_3$-[$^{32}$P]cAMP (150 µg, 7500 dpm), and then incubated (4 or 37°C, 5 or 60 min) in the absence or presence of an ATP-regenerating system (0.5 U creatine kinase, 10 mM creatine phosphate, 0.5 mM ADP) and LD, which had been prepared from GO-stimulated unlabelled adipocytes ($3.5 \times 10^4$ cells), in adiposome buffer supplemented with 0.2% (w/v) BSA (0.5 mL final vol.), as indicated. Thereafter, the complete incubation mixtures were suspended in 2.5 mL of 66% sucrose in phosphate buffered saline (PBS) containing 5 mM EDTA and transferred to the bottom of a centrifuge tube (SW41, Beckman). A 10–55% linear continuous sucrose density gradient containing 1 mM EDTA was layered on top (4.5 mL). After centrifugation (100 000× *g*, 16 h, 4°C), 0.5 mL fractions were collected from the bottom of the tube. Fractions were diluted with 4.5 mL of PBS containing 1 mM EDTA and then centrifuged (200 000× *g*, 1 h, 4°C). The pellets were washed once with 1 mL of adiposomes buffer.

*Miscellaneous*

Published procedures were used for the separation of rat adipocytes from the incubation medium (Müller *et al*., 1997), preparation of plasma membrane DIGs (Müller *et al*., 2001; 2002) and LD (Müller *et al*., 2008b) from rat adipocytes, cholesterol depletion of rat adipocytes by incubation (50 min, 30°C) in the absence or presence of 3.8 mM β-methylcyclodextrin (β-mCD) or 1.9 mM β-mCD *plus* 1.9 mM β-mCD-cholesterol complexes (Müller *et al*., 2002), measurement of cAMP-specific phosphodiesterase and 5′-nucleotidase activities (Müller *et al*., 2008d), assays for [$^{14}$C]palmitate- and [3-$^3$H]glucose-driven esterification (Müller *et al*., 2008c) and

glycerol and fatty acid release (Müller *et al.*, 2003), affinity purification of Gce1 and CD73 (Müller *et al.*, 2008a,d), determination of the adiposomal phosphatidylcholine (PC) content (Müller *et al.*, 2009), sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Müller *et al.*, 2001), extraction and precipitation of proteins from LD, DIGs, adiposomes and incubation medium under native and denaturing conditions (Müller *et al.*, 2008b,e) and determination of protein concentration using the bicinchoninic acid (BCA) method (Pierce) with BSA as calibration standard. Phosphorimages and lumiimages were processed and quantified by computer-assisted video densitometry using the Storm 860 PhosphorImager system (Molecular Dynamics, Gelsenkirchen, Germany) and LumiImager system (Roche Biochemicals, Mannheim, Germany) respectively. Concentration-response curves were fitted using the GraphPad Prism 4.03 software (GraphPad Software Inc., La Jolla, CA, USA). Figures of phosphoimages and lumiimages were constructed using the Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA).

*Statistical analysis*

Results are shown as mean ± SD. Differences between various treatments were analysed by unpaired Student's *t*-tests or one-way ANOVA as applicable with $P$ values <0.05 considered as significant.

*Materials*

*Myo*-[U-$^{14}$C]inositol was purchased from Amersham-Buchler (Braunschweig, Germany). [1-$^{14}$C]acetate was delivered by Biotrend (Cologne, Germany). Human recombinant insulin, glimepiride and GPI-2350 were made available by the biotechnology and medicinal chemistry departments of Sanofi-Aventis Pharma (Frankfurt, Germany). GO, pre-mixed protease (complete EASYpack) and phosphatase (PhosSTOP) inhibitor cocktails (1 tablet for 10 mL) were bought from Roche Molecular Biochemicals (Mannheim, Germany). Methylarachidonyl fluorophosphate (MAFP), arachidonyl trifluoromethylketone ($AACOCF_3$), pyrrolidine-2 (PY-2), bromoenol lactone (BEL), brefeldin-A (BFA), the inhibitor for $cPLA_2$ (CPLAI; Cat. No. 525143), cytochalasin D, monensin, nocodazole, orlistat, dinonylphthalate, β-methyl-cyclodextrin (β-mCD) and triacsin C were from Calbiochem-Merck (Darmstadt, Germany). β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine and β-D-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine were provided by Avanti Polar Lipids (Alabaster, AL, USA). Antibodies and all other (radio-labelled) materials were obtained as described previously (Müller and Wied, 1993; Müller *et al.*, 1997; 2001; 2002; 2005; 2008a,d).

## Results

*Differential expression of Gce1 and perilipin-A in adiposomes from large and small adipocytes*

GPI proteins share an evolutionarily conserved carboxy-terminal GPI glycolipid anchor that is generally assumed to target them to DIGs at the extracellular leaflet of the plasma membrane. In addition, in rat adipocytes challenged with physiological concentrations of palmitate and $H_2O_2$ or pharmacological concentrations of glimepiride, the GPI proteins Gce1 and CD73 have unexpectedly been found to be associated with intracellular LD, as well as to be released from the cells in adiposomes (Müller *et al.*, 2008a,b,c,e).

The signal-induced and selective expression of a GPI protein in PC-containing adiposomes is exemplified for Gce1 in response to GO (in the presence of glucose for the extracellular generation of $H_2O_2$ in the incubation medium). GO increased the amounts of Gce1 and PC recovered with the adiposomes by about 6- and 2.5-fold, respectively, leading to significant enrichment of Gce1 at PC-containing adiposomes compared to basal (Figure 1). In addition to GPI proteins, the LD-associated protein, perilipin A, and the milk fat globule-associated protein, milk fat globule membrane epidermal growth factor 8 (MFG-E8), have recently been identified as components of adiposomes upon exposure to various signals (Aoki *et al.*, 2007). In agreement, the levels of perilipin-A and MFG-E8 were elevated in adiposomes from GO-induced compared with basal adipocytes, albeit to a lower degree than Gce1, resulting in a low and no enrichment of perilipin-A and MFG-E8, respectively, versus PC. Thus, Gce1 and perilipin-A are incorporated into adiposomes in signal-induced fashion, whereas MFG-E8 and PC are constitutive adiposomal components, as is also reflected in the similar GO-induced enrichments of Gce1 or perilipin-A versus MFG-E8 and PC (Figure 1). Apparently, the expression of the GPI-protein, Gce1 and the LD-associated protein, perilipin-A, at adiposomes does not result in displacement of constitutive components, such as MFG-E8, from the ADIP, as revealed by its upregulation (GO induction) and downregulation (inhibition by GPI-2350, see below), which does not significantly affect the incorporation of MFG-E8 into the adiposomes. This is in agreement with our previous SDS-PAGE analyses (Müller *et al.*, 2009) comparing the total Coomassie-stained protein patterns of adiposomes released from signal-induced compared with basal adipocytes.

These and previous (Aoki *et al.*, 2007; Müller *et al.*, 2009) findings of adiposome release from adipocytes were performed with cells of large (>400 μm) to medium (150–300 μm) size. Interestingly, small adipocytes (<75 μm) separated from large ones by serial filtration through nylon mesh screens exhibited considerably less GO-induced, as well as basal release of adiposomes harboring Gce1, perilipin-A, MFG-E8 and PC (Figure 1). The resulting GO-induced enrichments of Gce1 versus MFG-E8 and PC compared with large adipocytes were rather moderate yet still significant. This differential responsiveness was observed on the basis of both identical lipid content contained in the large and small adipocyte populations, which resulted in an about 10- to 20-fold higher number of small versus large adipocytes (Figure 1) and the identical number of small and large adipocytes. This effect led to a considerably more pronounced difference in the release of Gce1- and perilipin-A-containing adiposomes (*per* cell) in favour of the latter (data not shown). The higher responsiveness of the signal-induced release of Gce1 and perilipin-A into MFG-E8- and PC-containing adiposomes in combination with their elevated constitutive release from large, compared with small, adipocytes suggests a differential function of ADIP-expressing GPI proteins and LD-associated proteins in adipocytes of different size.

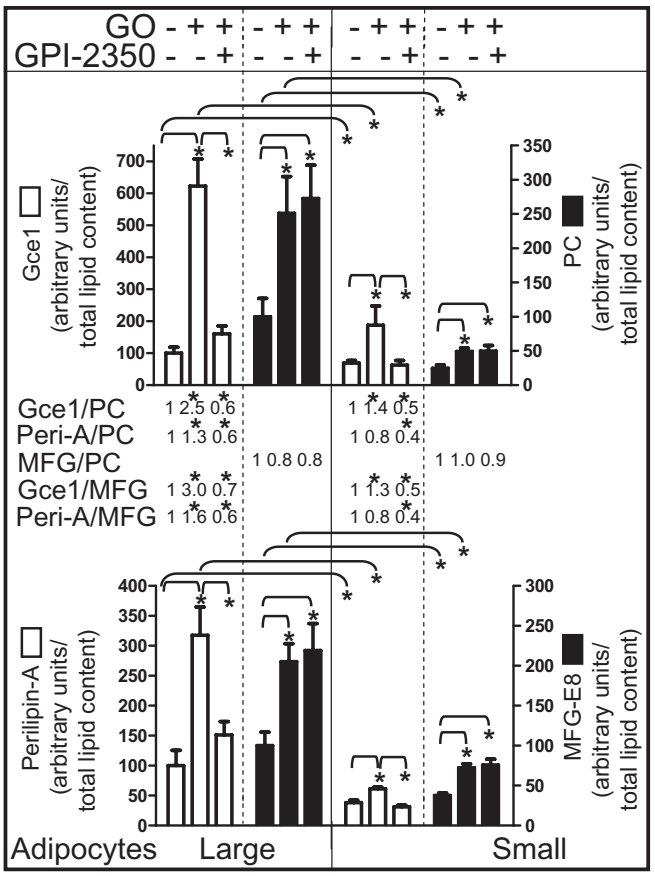


**Figure 1** Enrichment of Gce1, perilipin-A and milk fat globule membrane epidermal growth factor 8 (MFG-E8) in adiposomes released from large and small adipocytes. Isolated large or small rat adipocytes were incubated (37°C, 10 min) in the absence or presence of GPI-2350 (50 μM) prior to the addition of glucose oxidase (GO) (final conc. 500 mU·mL$^{-1}$) or buffer (basal). After further incubation (37°C, 30 min), adiposomes were prepared from the incubation media and extracted for proteins under native conditions, which were then analysed for Gce1 by photoaffinity labelling with 8-$N_3$-[$^{32}$P]cAMP, sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging, perilipin-A (Peri-A) and MFG-E8 (MFG) by immunoblotting and lumiimaging, and phosphatidylcholine (PC) by thin layer chromatography, staining and optical imaging. Quantitative evaluations of the images (mean ± SD from two adipocyte preparations with incubations in triplicate) are given as arbitrary units for identical total lipid contents of the large and small adipocyte suspensions set at 100 for Gce1, PC, perilipin-A and MFG-E8 each for the large adipocytes in the basal state, and marked with * for significant differences as indicated. The calculated ratios for the enrichment/depletion of Gce1, perilipin-A and MFG-E8 versus PC or MFG-E8 set at 1 for the basal state of large and small adipocytes, each are marked with * for significant differences versus the basal state.

*Sequential expression of Gce1, CD73 and cholesterol at DIGs, LD and adiposomes in response to palmitate, glimepiride and $H_2O_2$*

The apparent triple localization of Gce1 and CD73 at plasma membrane DIGs (in constitutive fashion), LD and adiposomes (in signal-induced fashion) may be based on independent direct routing to or redistribution between these sites. The critical role of DIGs in the biogenesis of GPI-protein-containing LD, as well as adiposomes, was demonstrated by interference with their formation. In order to demonstrate this, rat adipocytes were treated with a synthetic glycosphingolipid with non-natural stereochemistry, β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine. This compound has previously been shown to block the clustering of lipids and proteins in plasma membrane DIGs of human skin fibroblasts, HeLa and CV1 cells (Singh *et al.*, 2007). Thereafter, the release of the subsequently photoaffinity-labelled Gce1 and CD73 into adiposomes, as well as the inhibition of the isoprenaline-induced glycerol release – which reflects the translocation of Gce1 and CD73 from DIGs to LD and consequent downregulation of cAMP and lipolysis at the LD subcompartment (Müller *et al.*, 2008a,d) – in response to GO and glimepiride was assayed (Table 1). β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine, but not its natural stereoisomer, β-D-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine, which is known for a slight favoring of DIGs formation (Prudovsky *et al.*, 2008), significantly reduced the GO- and glimepiride-induced release of Gce1- and CD73-containing adiposomes, as well as the inhibition of isoprenaline-stimulated lipolysis. Thus, it seems that the integrity of plasma membrane DIGs was required for the induced translocation to LD, as well as the release into adiposomes of Gce1 and CD73, which had been labelled at the cell surface. This result is compatible with a direct function of plasma membrane DIGs in the release of GPI proteins into LD and adiposomes in either simultaneous or consecutive fashion.

In order to discriminate between these possibilities, Gce1 and CD73 were pulse labelled by photoaffinity labelling of their protein moieties with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-FSBA, respectively (Figure 2), or by metabolic labelling of their GPI anchors with *myo*-[$^{14}$C]inositol (Figure 3A and B), or labelled for long term with *myo*-[$^{14}$C]inositol (Figure 3C and D). After incubation under chase conditions in the absence or presence of palmitate, glimepiride and GO, the distributions of the photoaffinity-labelled (Figure 2) or metabolically labelled and subsequently affinity-purified (Figure 3) Gce1 and CD73 between DIGs, LD and adiposomes were analysed by cell fractionation. In the basal state, the amounts of pulse-labelled Gce1 and CD73 at the DIGs were highest at the zero time point (Figure 2 for photoaffinity labelling) or 5–15 min later (Figure 3A and B for metabolic labelling), and subsequently steadily decreased with time. In contrast, in the LD, they increased from very low levels at the zero time point to maximal levels at 30–60 min with subsequent sharp declines. In the adiposomes, pulse-labelled Gce1 and CD73 were hardly detectable at the zero time point and then continuously increased with the chase time (Figures 2 and 3A and B). Challenge of the adipocytes with maximally effective concentrations of palmitate, glimepiride and GO did not affect the shape of the time courses but significantly increased the rates of disappearance of pulse-labelled Gce1 and CD73 at the DIGs, as well as the appearance at both LD (up to the peak levels) and adiposomes (Figure 2 for photoaffinity labelling; Figure 3A and B for metabolic labelling) with the ranking order in potency of GO > glimepiride > palmitate.

Metabolic labelling of Gce1 and CD73 for long term resulted in similar time courses for each location and induction as observed for the pulse labelling, with the exception that it led to continuous declines rather than peak levels at the DIGs and to earlier peak levels at the LD during the chase

**Table 1** Effect of dissociation of plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) on the release of adiposomes and inhibition of lipolysis by glucose oxidase (GO) and glimepiride in rat adipocytes

| | *[μM]* | *Adiposome release (control set at 100)* | | | | *Lipolysis inhibition (control set at 100)* | |
|---|---|---|---|---|---|---|---|
| | | *GO* | | *Glimepiride* | | *GO* | *Glimepiride* |
| | | *Gce1* | *CD73* | *Gce1* | *CD73* | | |
| Control | – | 100 ± 31 | 100 ± 35 | 100 ± 28 | 100 ± 23 | 100 ± 12 | 100 ± 14 |
| L-*t*-LacCer | 3 | 62 ± 20 | 51 ± 15* | 68 ± 19 | 59 ± 15* | 43 ± 9* | 47 ± 12* |
| | 30 | 28 ± 9* | 15 ± 6* | 32 ± 13* | 18 ± 6* | 5 ± 4* | 3 ± 2* |
| D-*e*-LacCer | 3 | 106 ± 20 | 112 ± 29 | 83 ± 27 | 96 ± 30 | 104 ± 9 | 97 ± 13 |
| | 30 | 93 ± 25 | 91 ± 22 | 89 ± 26 | 88 ± 19 | 95 ± 13 | 90 ± 8 |

Rat adipocytes were incubated (37°C, 20 min) in the absence (Control) or presence of β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine (L-*t*-LacCer) or β-D-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine (L-*e*-LacCer) complexed to defatted bovine serum albumin (BSA). For studying adiposome release, portions of the cells were photoaffinity-labelled with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-FSBA and then incubated (37°C, 4 h) in the absence (basal) or presence of GO (500 mU·mL$^{-1}$) or glimepiride (10 μM). Adiposomes were prepared from the incubation media and extracted for proteins under denaturing conditions, which were then analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging. For studying lipolysis inhibition, other portions of the adipocytes were incubated (37°C, 20 min) in the absence (basal) or presence of GO (500 mU·mL$^{-1}$) or glimepiride (10 μM). After further incubation (37°C, 4 h) with isoprenaline (1 μM), the incubation media were analysed for glycerol. Quantitative evaluations (mean ± SD from four adipocyte preparations with incubations in duplicate) are given with the increases in the amounts of Gce1-/CD73-containing adiposomes and the declines in the isoprenaline -stimulated glycerol release (lipolysis inhibition) versus basal set at 100 for each induction in the absence of glycosphingolipids (Control) and marked with *for significant differences versus the corresponding control induction.

period (Figure 3C and D). The amounts of metabolically labelled Gce1 and CD73 in the adiposomes were continuously up-regulated. Again, GO, glimepiride and palmitate – in that order of decreasing potency – significantly accelerated compared with basal, the declines of Gce1 and CD73 at the DIGs, as well as their elevations in the LD (to up to peaking at 25–30 min) and adiposomes (to up to 120 min). Taken together, the precursor-product relationship between Gce1/CD73 located at DIGs and Gce1/CD73 located at LD during 0–30 min, as well as between Gce1/CD73 at LD and adiposomes during 30–120 min [data derived from both pulse (photoaffinity and metabolic) and long-term (metabolic) labelling] suggest a sequential expression of Gce1 and CD73 at DIGs, LD and adiposomes, which is stimulated by $H_2O_2$ > glimepiride > palmitate.

In addition to GPI proteins, cholesterol is known to be enriched at plasma membrane DIGs and LD compared with total membranes in adipocytes (Dugail *et al*., 2003). Therefore, the redistribution of [$^3$H]cholesterol between DIGs, LD and adiposomes was studied after pulse chase or long-term labelling under the conditions used for the analysis of Gce1 and CD73 (Figure 4). After pulse and long-term labelling of basal adipocytes, maximal levels of cholesterol at the DIGs were observed at 30 min and at the zero time point respectively. During the subsequent chase of both pulse and long-term labelling, cholesterol continuously declined in the DIGs and increased in the LD at rates which were each considerably increased compared with basal by GO, glimepiride and palmitate in that order of declining potency. In contrast, only minute amounts of cholesterol accumulated during the chase in the adiposomes, with no significant differences between basal and induced adipocytes (Figure 4). Thus, the precursor-product relationship between DIGs- and LD-associated cholesterol, as revealed by the pulse and long-term labelling, is compatible with the concept of translocation of cholesterol from DIGs to LD, which is stimulated by $H_2O_2$ > glimepiride > palmitate. In contrast, the incorporation of major amounts of cholesterol into adiposomes seems unlikely.

### *Role of $H_2O_2$ production, GPI-protein translocation, cholesterol and vesicular transport in the release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes*

The above results suggest that the drug-stimulated translocation of Gce1 and CD73 from plasma membrane DIGs to LD precedes their incorporation into adiposomes. Previous studies have revealed that the translocation of Gce1 and CD73, as well as of caveolin-1 and perilipin-A, from plasma membrane DIGs to LD critically depends on the (i) production of $H_2O_2$ by a NADPH oxidase, (ii) activation of a GPI-specific phospholipase C (GPI-PLC), which cleaves GPI anchors, (iii) absence of non-cleavable substrate analogues for the enzymic activity of Gce1 or CD73; and (iv) presence of DIGs (Müller *et al*., 2001; 2002; 2008a,b,c,e). Consequently, the requirement of the translocation of Gce1, CD73, caveolin-1 and perilipin-A for their release into adiposomes was studied by using the following agents: $H_2O_2$-degrading catalase; the inhibitor of NADPH oxidases, diphenylene iodonium (DPI), the inhibitor of the GPI-PLC, GPI-2350 (Müller *et al*., 2005), and the non-hydrolyzable AMP analogue, α,β-methylene-ADP (AMPCP) (Figure 5). Disruption of the DIGs by cholesterol depletion was also evaluated (Figure 6).

Catalase, GPI-2350 and AMPCP significantly reduced the GO-, glimepiride- and palmitate-induced levels of photoaffinity-labelled Gce1 and CD73. Immunoblotted caveolin-1 and perilipin-A (Figures 1 and 5A), as well as of the cAMP-specific phosphodiesterase and 5′-nucleotidase activities of Gce1 and CD73 (Figure 5B) associated with adiposomes, were also affected. Noticably, GPI-2350 specifically interferred with signal-induced adiposome expression of Gce1 and perilipin-A, but did not affect the (constitutive and

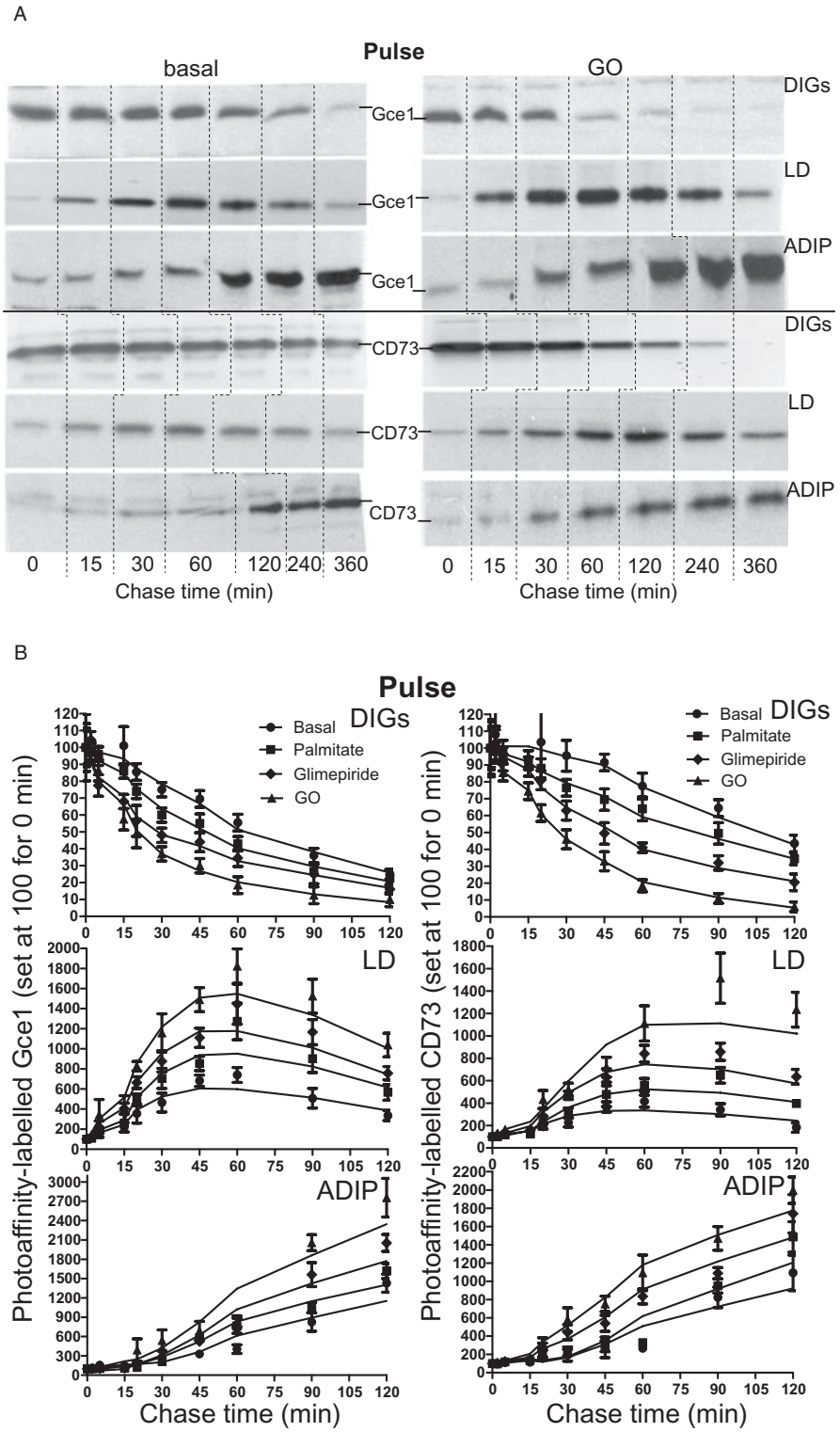


**Figure 2** Translocation of photoaffinity-labelled Gce1 and CD73 from plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) to lipid droplets (LD) and adiposomes (ADIP). Isolated rat adipocytes were photoaffinity labelled with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-FSBA (Pulse), washed and then incubated (37°C) for increasing periods of (chase) time in the absence (basal) or presence of GO (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM) or palmitate (1 mM). Thereafter, plasma membrane DIGs, LD and adiposomes were prepared from the adipocytes and incubation media, respectively, and extracted for proteins under denaturing conditions, which were then analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging. Representative phosphorimages for basal and GO-treated cells are shown (A) with quantitative evaluations (means ± SD from three adipocyte preparations with incubations in duplicate) set at 100 for DIGs, LD and adiposomes each at chase time point zero (B).

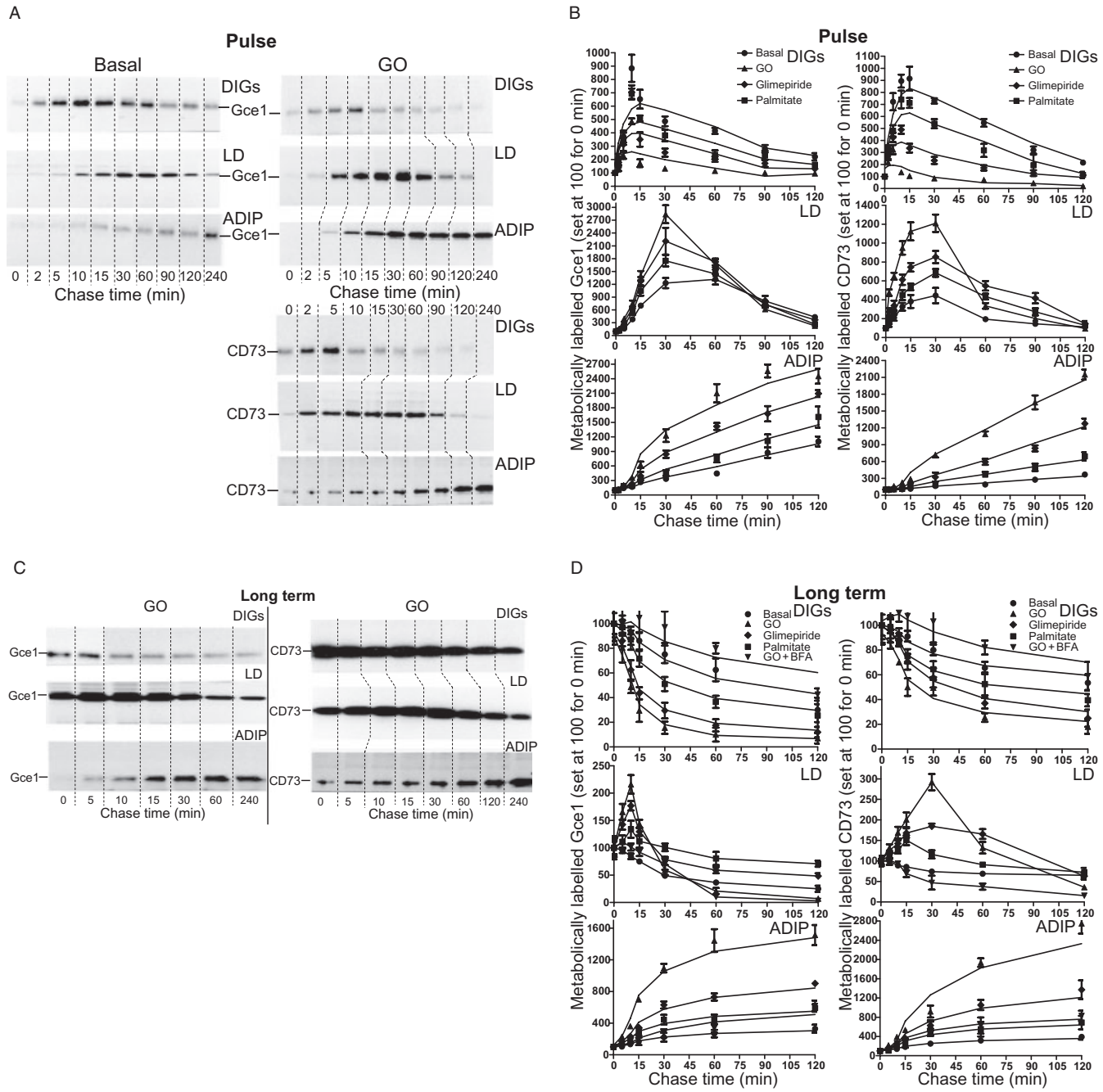


**Figure 3** Translocation of metabolically labelled Gce1 and CD73 from plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) to lipid droplets (LD) and adiposomes (ADIP). Isolated rat adipocytes were metabolically labelled with *myo*-[$^{14}$C]inositol for 10 min (Pulse; A and B) or 5 h (Long term; C and D) and then incubated (37°C) with excess of *myo*-inositol for increasing periods of (chase) time in the absence (basal) or presence of GO (final conc. 500 mU·mL$^{-1}$) without or with brefeldin-A (final conc. 5 μg·mL$^{-1}$, D only), glimepiride (10 μM) or palmitate (1 mM). Thereafter, plasma membrane DIGs, LD and adiposomes were prepared from the adipocytes and incubation media, respectively, and extracted for proteins under native conditions, which were then adsorbed to cAMP sepharose. Affinity-purified Gce1 and CD73 were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging. Representative phosphorimages for basal and GO-treated cells are shown (A and C) with quantitative evaluations (mean ± SD from 2 adipocyte preparations with incubations in triplicate) set at 100 for DIGs, LD and ADIP each at chase time point zero (B and D).

signal-induced) formation of MFG-E8- and PC-harboring adiposomes (Figure 1). Thus, GPI-PLC action is apparently required for the release of GPI proteins and LD-associated proteins into adiposomes, but not for their formation *per se*. However, the GPI-PLC-dependent GPI protein expression in the adiposomes *per se* does not affect the (constitutive as well as signal-induced) release of adiposomes. DPI blocked the glimepiride- and palmitate- but not GO-induced upregulation of Gce1, CD73, caveolin-1 and perilipin-A, as well as Gce1/CD73 activities in adiposomes (Figure 5). This result suggest that there is a requirement of NADPH oxidase-driven $H_2O_2$ production for the release of GPI protein- and LD-associated

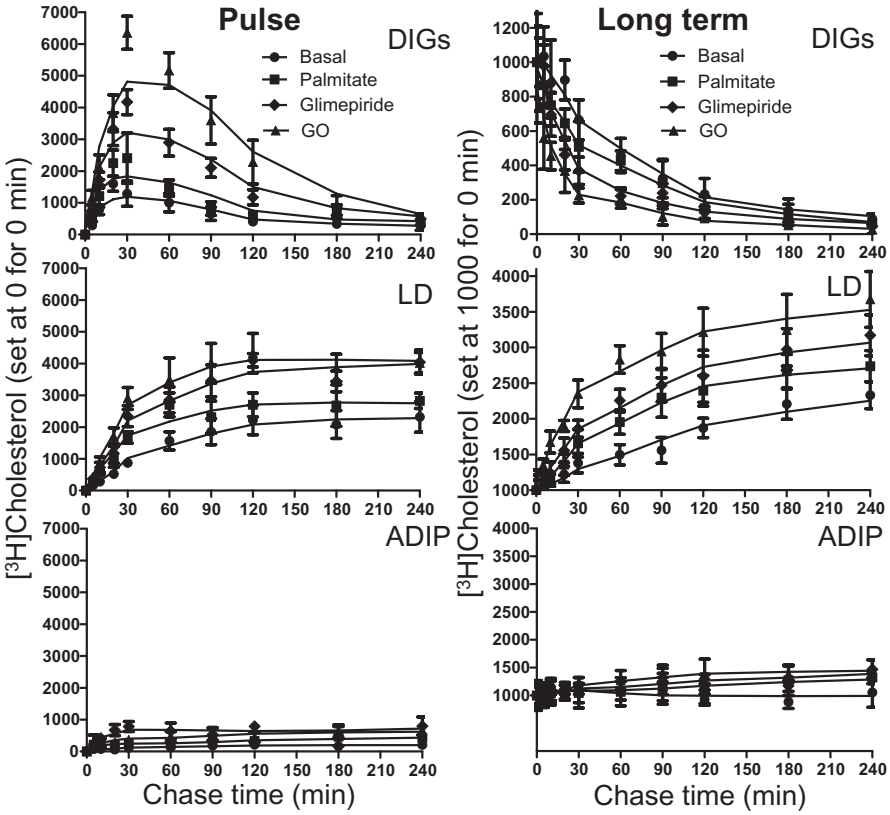


**Figure 4** Translocation of cholesterol from plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) to lipid droplets (LD). Isolated rat adipocytes were metabolically labelled with [$^{14}$C]acetate for 5 min (Pulse) or 1 h (Long term) and then incubated (37°C) with excess of acetate (5 mM) for increasing periods of (chase) time in the absence (basal) or presence of GO (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM) or palmitate (1 mM). Thereafter, plasma membrane DIGs, LD and adiposomes (ADIP) were prepared from the adipocytes and incubation media, respectively, and extracted for cholesterol, which was then analysed by thin layer chromatography and phosphorimaging. Quantitative evaluations (mean ± SD from 4 adipocyte preparations with incubations in duplicate) are given set at 0 (Pulse) and 1000 (Long term), respectively, for DIGs, LD and ADIP each at chase time point zero.

protein-harbouring adiposomes in response to glimepiride and palmitate, which is bypassed by exogenous GO action.

The effect of disruption of the plasma membrane DIGs was studied with basal and induced rat adipocytes, which were then metabolically labelled with *myo*-[$^{14}$C]inositol before or after cholesterol depletion in the course of its complexing with β-mCD (Figure 6). After 2 or 10 min of chase with unlabelled inositol, only low amounts of Gce1 (Figure 6A) and CD73 (Figure 6B) metabolically labelled either before or after the m-βCD treatment were detected at the LD of cholesterol-depleted compared with cholesterol-containing GO-, glimepiride- or palmitate-induced cells. Moreover, depletion before the labelling significantly reduced the GO-induced loss of Gce1 and CD73 from the DIGs. In contrast, cholesterol depletion after the metabolic labelling of induced adipocytes drastically increased the GO-, glimepiride- or palmitate-induced levels of Gce1 and CD73 recovered with the adiposomes in chase in a time-dependent fashion. During these short chase periods only minute amounts of Gce1 and CD73 were released into adiposomes from non-depleted induced or basal adipocytes. Increasing of the chase from 10 min to up to 120 min revealed that the losses of Gce1 and CD73 from LD and upregulation at adiposomes were significantly enhanced in response to GO, glimepiride and palmitate and thereby compromised the corresponding effects of the β-mCD treatment (data not shown). Apparently, the relative potency of the three stimuli (GO > glimepiride > palmitate) in inducing the rapid loss from the LD and gain by the adiposomes of the GPI proteins in cholesterol-depleted adipocytes (within minutes, see Figure 6) was the same as observed for the corresponding slower effects in cholesterol-containing cells (within hours, see Figure 2). The specificity of the cholesterol depletion was confirmed by the simultaneous presence of β-mCD and cholesterol either before or after the metabolic labelling, which had no effect on the induced redistribution of Gce1 and CD73 between DIGs, LD and adiposomes during the subsequent chase (Figure 6).

Taken together, disruption of plasma membrane DIGs by cholesterol depletion (Figure 6) or β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine treatment (Table 1) prior to metabolic or photoaffinity labelling of Gce1 and CD73 blocks their translocation to LD, and, in consequence, their incorporation into adiposomes. Thus, cholesterol-containing functional DIGs seem to be required for the translocation of Gce1, CD73, perilipin-A and caveolin-1 to the LD, and may operate as the source for the cholesterol accompanying these proteins during their translocation from plasma membrane DIGs to LD (see Figure 4). In contrast, depletion of cholesterol from LD – which is a consequence of m-βCD treatment of intact adipocytes due to the mutual exchange of cholesterol between

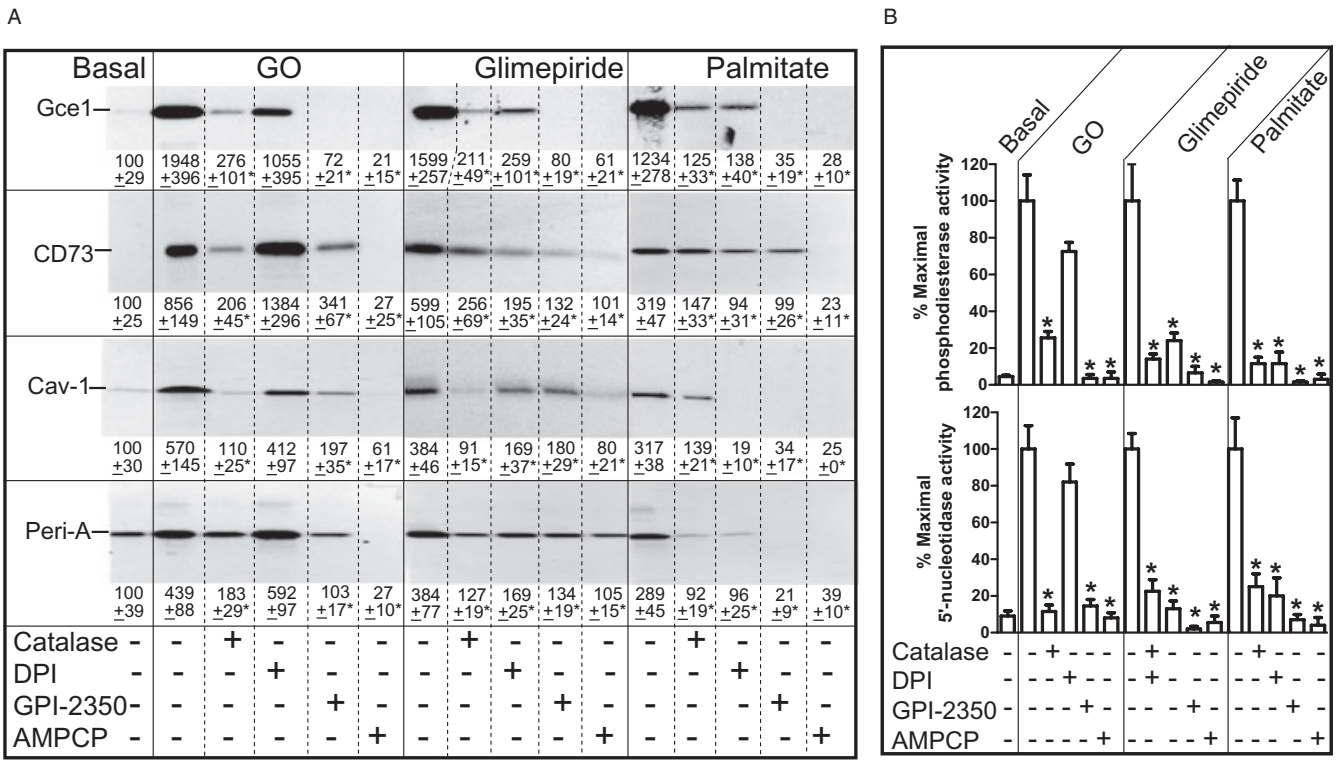


**Figure 5** Effect of inhibition of the translocation of Gce1, CD73, caveolin-1 and perilipin-A from plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) to lipid droplets (LD) on their release into adiposomes. Isolated rat adipocytes photoaffinity-labelled with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C] 5′-*p*-fluorosulphonylbenzoyladenosine (A) or unlabelled (B) were incubated (37°C, 10 min) in the absence or presence of catalase (1 U·mL$^{-1}$), diphenylene iodonium (DPI) (100 μM), (GPI-2350 is the inhibitor; see *Materials*) (50 μM) or α,β-methlyene-ADP (AMPCP) (100 μM) prior to the addition of glucose oxidase (GO) (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM), palmitate (1 mM) or buffer (basal). After further incubation (37°C, 30 min), adiposomes were prepared from the incubation media and extracted for proteins under native conditions, which were then analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging (A), caveolin-1 (Cav-1) and perilipin-A (Peri-A) by immunoblotting and lumiimaging (A) and cAMP-specific phosphodiesterase and 5′-nucleotidase activities (B). Representative phosphor-/lumi-images (A) or activity measurements (B) are shown with quantitative evaluations (mean ± SD from three adipocyte preparations with incubations in duplicate) set at 100 in the absence of inhibitor for basal (A) and induced (B) adipocytes each, and marked with * for significant differences versus the correspondingly induced adipocytes in the absence of inhibitor.

plasma membrane DIGs and LD – favours the translocaton of Gce1 and CD73 from LD into adiposomes. This was monitored by metabolic labelling prior to the m-βCD treatment and the resultant signal-induced accumulation of labelled GPI proteins at the LD during the period before disruption of the plasma membrane DIGs and blockade of the GPI protein translocation (Figure 6). LD (from untreated adipocytes) seem to retain GPI proteins and to prevent their release into adiposomes unless they have been depleted of cholesterol.

In conclusion, these findings suggest that cholesterol accompanies and thereby supports the translocation of Gce1, CD73, caveolin-1 and perilipin-A from plasma membrane DIGs to LD, and is a prerequisite for their subsequent signal-induced release into adiposomes, which, however, is impaired by cholesterol on arrival at the LD. This dual role of cholesterol explains the apparent differential effects of its depletion at plasma membrane DIGs and LD on the expression of Gce1, CD73, caveolin-1 and perilipin-A at LD (inhibition) and adiposomes (stimulation) respectively.

The initial characterization of the molecular mechanisms involved in the translocation to LD and release into adiposomes of Gce1, CD73, caveolin-1 and perilipin-A, and the effects of typical inhibitors of cytoskeleton assembly and vesicular transport (Dinter and Berger, 1998; Prudovsky *et al*., 2008), were analysed (Figure 7). The GO-, glimepiride- and palmitate-induced expression of photoaffinity-labelled Gce1 and CD73 (Figure 7A), as well as immunoblotted caveolin-1 in both LD and adiposomes and perilipin-A in adiposomes, but not in LD (Figure 7B), was significantly reduced (sometimes below the basal level) in the presence of maximally effective concentrations of the microtubule-disrupting reagent, nocodazole, and the Golgi apparatus-perturbing reagents, monensin and BFA. In agreement with these results is the finding of retention or even accumulation above basal levels of Gce1 and CD73 at plasma membrane DIGs of GO-induced and metabolically labelled (long term) adipocytes in the presence of BFA, with concomitant blockade of their translocation to LD and release into adiposomes during the complete chase period (Figure 3D). Moreover, in adipocytes incubated with 2-deoxyglucose *plus* $NaN_3$ (known to cause efficient ATP depletion in rat adipocytes) or with the endocytosis inhibitor methylamine, but not with the actin filament-disrupting reagent cytochalasin D, the amounts of photoaffinity-labelled Gce1 recovered

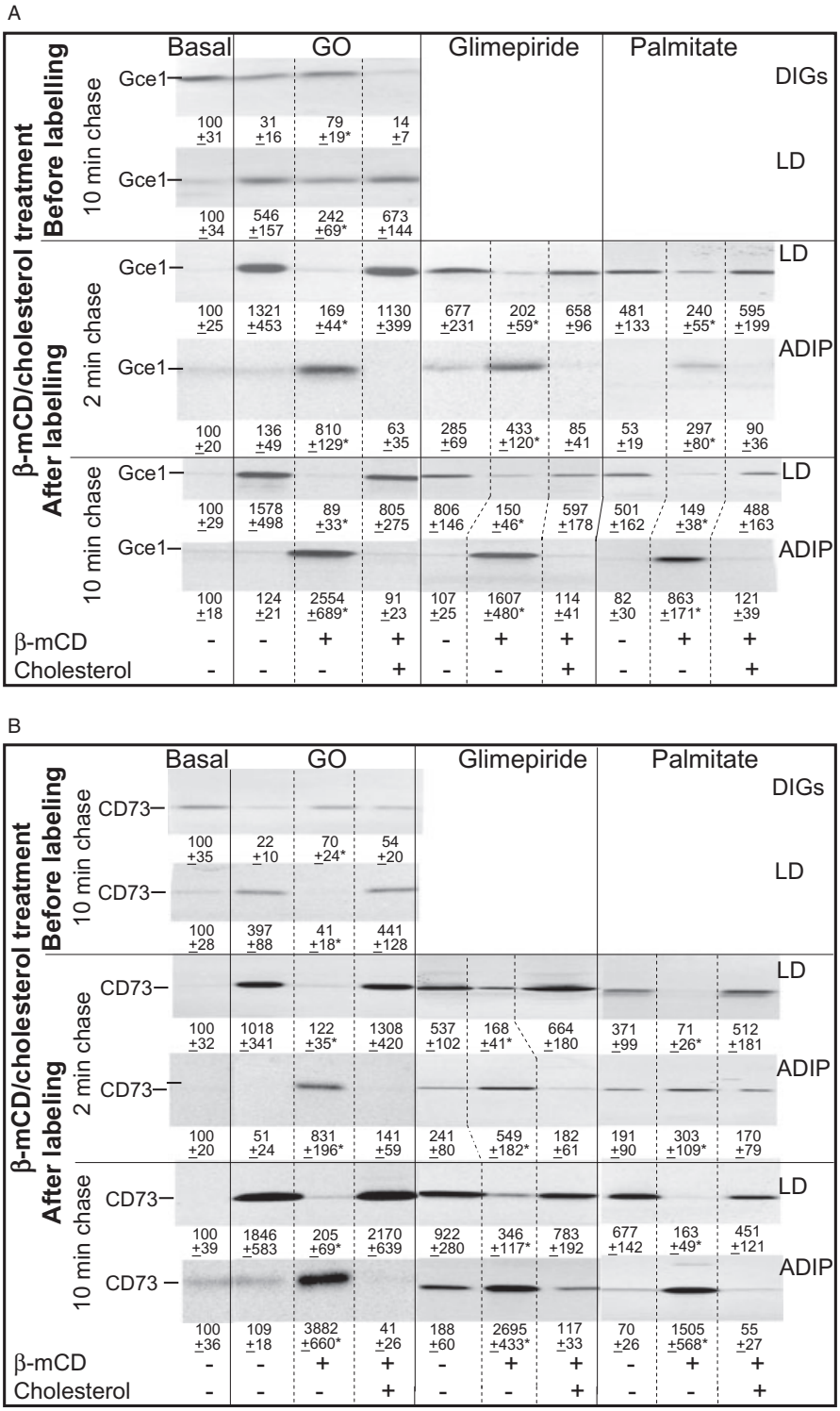


**Figure 6** Effect of cholesterol depletion on the translocation of Gce1 and CD73 from plasma membrane (detergent-insoluble glycolipid-enriched plasma membrane microdomains) DIGs *via* lipid droplets (LD) to adiposomes (ADIP). Isolated rat adipocytes were incubated (20 min, 37°C) in the absence (basal) or presence of glucose oxidase (GO) (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM) or palmitate (1 mM) and then treated with β-mCD, cholesterol or buffer before or after metabolic labelling (10 min) with [$^{14}$C]inositol. Following addition of unlabelled *myo*-inositol (final conc. 10 mM), the incubation was continued (2 or 10 min chase). Thereafter, plasma membrane DIGs, LD and adiposomes were prepared from the adipocytes and incubation media, respectively, and extracted for proteins under native conditions, which were then adsorbed to cAMP sepharose. Affinity-purified Gce1 (A) and CD73 (B) were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging. Representative phosphorimages are shown with evaluations (mean ± SD from three adipocyte preparations with incubations in duplicate) set at 100 for DIGs, LD and adiposomes from basal adipocytes and marked with * for significant differences versus the correspondingly induced adipocytes in the absence of β-mCD and cholesterol.

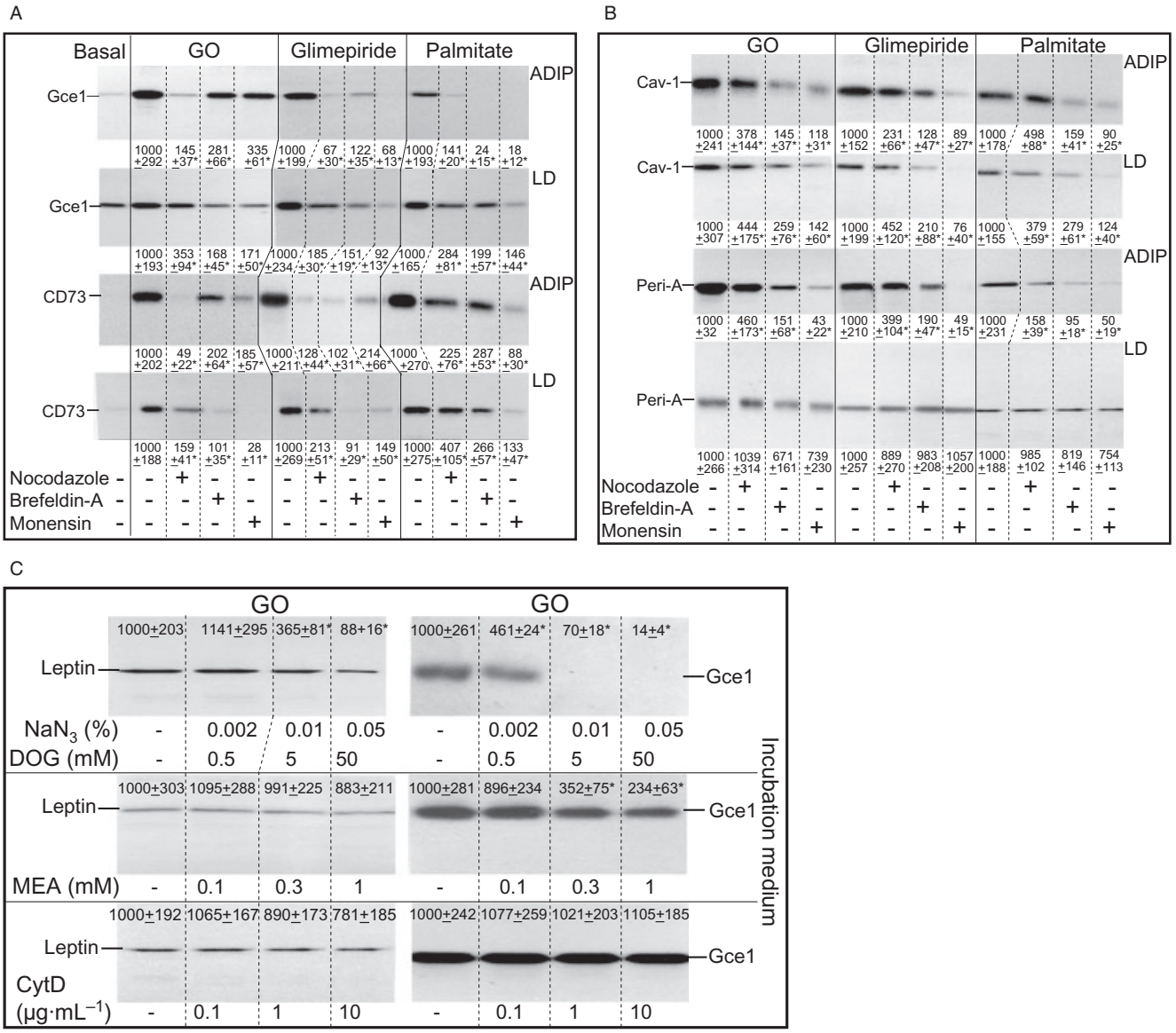


**Figure 7** Effect of inhibition of the secretory pathway on the release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes (ADIP). Isolated rat adipocytes were photoaffinity labelled with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-*p*-fluorosulphonylbenzoyladenosine and then incubated (37°C, 5 min) in the absence or presence of nocodazole (final conc. 20 μM), brefeldin-A (5 μg·mL$^{-1}$) and monensin (50 μM) (A and B) or $NaN_3$ *plus* 2-deoxyglucose (DOG), methylamine (MEA) and cytochalasin D (CytD) at increasing concentrations (C) prior to the addition of glucose oxidase (GO) (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM), palmitate (1 mM) or buffer (basal). After further incubation (37°C, 120 min), the adipocytes (top) were separated from the incubation media (infranatant) by flotation (200× *g*, 2 min, 20°C). From portions of the incubation media, adiposomes were prepared (A and B), or total proteins were precipitated under denaturing conditions (C). From the adipocytes, lipid droplets (LD) were prepared (A and B). Adiposomes and LD were extracted for proteins under denaturing conditions and then analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging (A), or caveolin-1 (Cav-1) and perilipin-A (Peri-A) by immunoblotting and lumi-imaging (B). Other portions of the incubation media were analysed for leptin by immunoblotting and lumi-imaging (C). Representative phosphorimages (A) and lumi-images (B and C) are shown with quantitative evaluations (mean ± SD from four adipocyte preparations with incubations in duplicate) set at 1000 for each induction in the absence of inhibitor and marked with * for significant differences versus the correspondingly induced adipocytes in absence of inhibitor.

from the total incubation medium (including adiposomes) of GO-induced adipocytes were diminished, concentration dependently (Figure 7C). The secretion of the soluble adipocytokine leptin, which is known to rely on the classical vesicular pathway (Bradley *et al*., 2001), was maintained in the presence of methylamine and cytochalasin D, but decreased in the presence of 2-deoxyglucose *plus* $NaN_3$ (Figure 7C). Significantly, there was no reduction in viability of the adipocytes under these conditions as measured by both propidium iodide staining and release of lactate dehydrogenase into the incubation medium (data not shown). These findings strongly imply that the translocation of GPI proteins to LD and their release into adiposomes are ATP dependent and require Golgi apparatus-, endosome- and microtubule-dependent steps. Golgi apparatus and microtubules seem also to be involved in the translocation and

release of caveolin-1, but only in the adiposome release of perilipin-A.

*Role of the LD content of adipocytes on the expression of Gce1, CD73, caveolin-1 and perilipin-A at LD and adiposomes in response to palmitate, glimepiride and $H_2O_2$*

LD are the major storage compartments of free cholesterol in adipocytes (Dugail *et al.*, 2003). Therefore, the size and/or number of the LD may play a modulatory role in the GO-, glimepiride- and palmitate-induced translocation of Gce1, CD73, caveolin-1 and perilipin-A from the plasma membrane DIGs to adiposomes. In order to evaluate this relationship, the LD content of adipocytes was up-regulated by long-term incubation with high concentrations of insulin or glucose, both of which cause marked stimulation of synthesis and block lipolytic degradation of TAG in adipocytes. (Müller *et al.*, 1994). Insulin and glucose significantly compromised the GO-, glimepiride- and palmitate-induced expression of photoaffinity-labelled Gce1 and CD73, as well as immunoblotted caveolin-1 and perilipin-A in adiposomes to basal or even below basal levels (Figure 8).

Conversely, the LD content of adipocytes was down-regulated in response to the β-adrenoceptor agonist isoprenaline, which causes lipolytic degradation and blockade of TAG synthesis (Figure 9). Long-term incubation with isoprenaline significantly increased the GO-, glimepiride- and palmitate-induced levels of photoaffinity-labelled Gce1 and CD73 proteins (Figure 9A), as well as cAMP-specific phosphodiesterase and 5′-nucleotidase activities (Figure 9B) recovered in the adiposomes compared with control cells. The effects of isoprenaline were reversed in the presence of orlistat, an inhibitor of the major TAG-degrading enzymes, hormone-sensitive lipase and adipocyte triglyceride lipase, in rodent adipocytes (Müller and Petry, 2005; Watt and Steinberg, 2008).

At the LD, the induced abundance of photoaffinity-labelled Gce1 and CD73 was significantly diminished in isoprenaline-treated compared with control and isoprenaline plus orlistat-treated cells (Figure 9A). The amounts of immunoblotted caveolin-1 and perilipin-A were significantly reduced in the adiposomes and LD after the long-term incubation of GO-, glimepiride- and palmitate-induced adipocytes with isoprenaline, but not isoprenaline plus orlistat (Figure 9C). The relative potency of GO > glimepiride > palmitate in inducing the translocation to LD and release into adiposomes of Gce1, CD73, caveolin-1 and perilipin-A was identical for adipocytes with elevated (insulin, high glucose; Figure 8) and diminished (isoprenaline; Figure 9) LD content. The efficacy of the long-term incubation of the adipocytes with isoprenaline in reducing the amounts of intracellular TAG and LD was reflected in

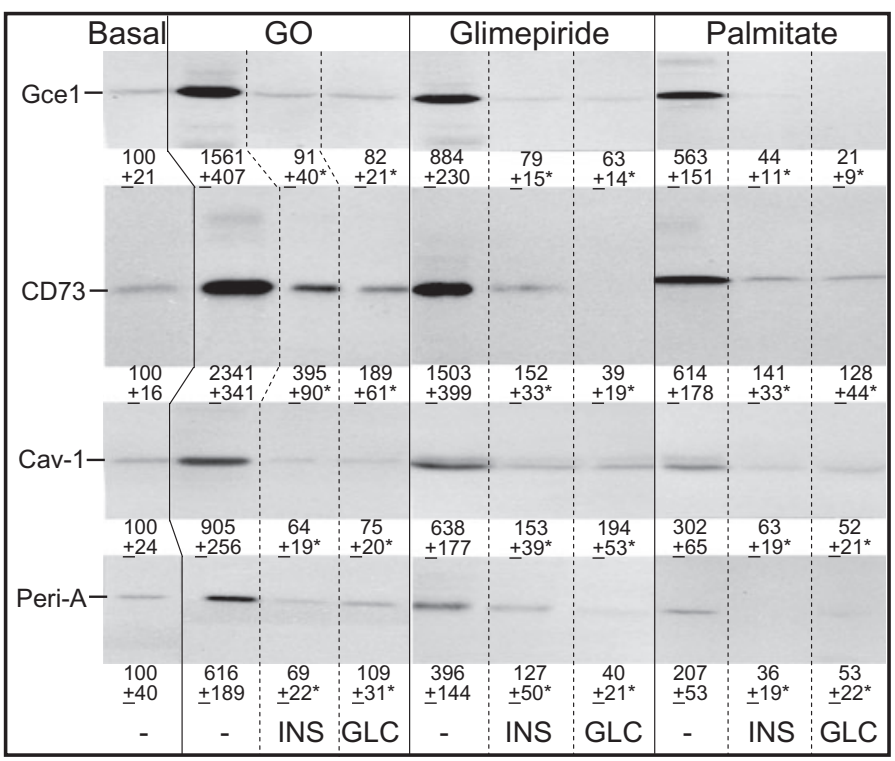


**Figure 8** Effect of increased lipid droplets (LD) content on the release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes. Isolated rat adipocytes were incubated (37°C, 18 h) in primary culture in the absence or presence of insulin (10 nM, INS) or glucose (20 mM, GLC) prior to the addition of glucose oxidase (GO) (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM), palmitate (1 mM) or buffer (basal). After further incubation (37°C, 4 h), adiposomes were prepared from the incubation media and then photoaffinity labelled with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-*p*-fluorosulphonylbenzoyladenosine. Proteins were extracted under denaturing conditions and then analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging or caveolin-1 (Cav-1) and perilipin-A (Peri-A) by immunoblotting and lumi-imaging. Representative phosphor-/lumiimages are shown with quantitative evaluations (mean ± SD from two adipocyte preparations with incubations in triplicate) set at 100 for basal adipocytes in the absence of insulin or glucose and marked with * for significant differences versus the correspondingly induced adipocytes in the absence of insulin or glucose.

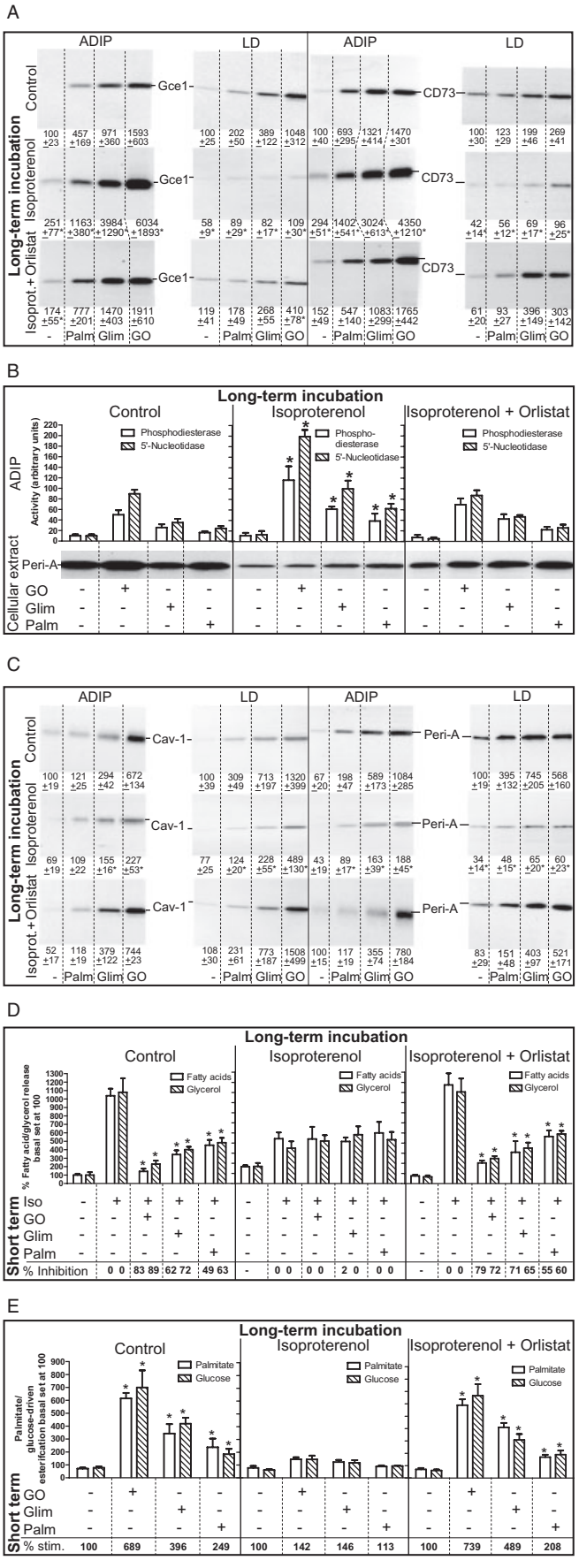


**Figure 9** Effect of decreased lipid droplets (LD) content on the release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes (ADIP). Isolated rat adipocytes were incubated (Long-term incubation; 37°C, 18 h) in primary culture in the absence (Control) or presence of isoprenaline (final conc. 1 μM) and orlistat (100 μM). Portions of the adipocytes were photoaffinity labelling with 8-$N_3$-[$^{32}$P]cAMP or [$^{14}$C]5′-*p*-fluorosulphonylbenzoyladenosine (A and C). Other portions were left untreated for subsequent assaying of cAMP-specific phosphodiesterase and 5′-nucleotidase activities (B) or fatty acid and glycerol release (D) or palmitate- and glucose-driven esterification (E). Each portion of the adipocytes was incubated (Short term; 37°C, 60 min) without (A, B, C and E) or with (D) isoprenaline (Iso, final conc. 1 μM) in the absence (basal) or presence of glucose oxidase (GO) (500 mU·mL$^{-1}$), glimepiride (Glim, 10 μM) or palmitate (Palm, 1 mM) as indicated. The incubation media (infranatant) were separated from the adipocytes (top) by flotation (200× *g*, 2 min, 20°C). Thereafter, adiposomes and LD were prepared from the incubation media and adipocytes, respectively, and then extracted for proteins under native conditions (A, B and C). Portions were analysed for Gce1 and CD73 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging (A) or caveolin-1 (Cav-1) and perilipin-A (Peri-A) by immunoblotting and lumiimaging (C). Representative phosphorimages (A) and lumi-images (C) are shown with quantitative evaluations for basal adipocytes from the control incubation set at 100 and marked with * for significant differences versus the correspondingly induced control adipocytes. Portions of the adiposomes were analysed for cAMP-specific phosphodiesterase and 5′-nucleotidase activities with quantitative evaluations (mean ± SD from two adipocyte preparations with incubations in duplicate) set at 10 for basal control adipocytes and marked with * for significant differences versus the correspondingly induced control adipocytes (B). Cellular extracts were prepared from the adipocytes (identical amounts of protein), extracted for proteins under denaturing conditions and then analysed for perilipin A (Peri-A) by immunoblotting and lumi-imaging (B). Incubation media were analysed for fatty acids and glycerol (D) with quantitative evaluations (mean ± SD from three adipocyte preparations with incubations in quadruplicate) set at 100 for basal (Short term) control (Long-term incubation) adipocytes and marked with * for significant differences versus the isoprenaline-induced (Short term) correspondingly incubated (Long-term incubation) adipocytes. Inhibition percentage is set at 0 for the isoprenaline-induced (Short term) correspondingly incubated (Long-term incubation) adipocytes. From other portions of the adipocytes, esterification of [$^{14}$C]palmitate or [$^{3}$H]glucose into triacylglycerol was assayed (E) with quantitative evaluations (mean ± SD from two adipocyte preparations with incubations in triplicate) set at 100 for basal (Short term) control (Long-term incubation) adipocytes and marked with * for significant differences versus the basal (Short term) correspondingly incubated (Long-term incubation) adipocytes. Stimulation percentage is set at 100 for the basal (Short term) correspondingly incubated (Long-term incubation) adipocytes.

the considerably lowered levels of the typical LD marker and PAT protein, perilipin-A, in total cellular extracts prepared from identical numbers of isoprenaline -treated compared with control and isoprenaline plus orlistat-treated cells (Figure 9B). As expected, the lipolytic loss of TAG and LD during the long-term isoprenaline incubation was not compensated in the subsequently GO-, glimepiride- and palmitate-induced adipocytes in course of the short-term inhibition of lipolysis. These data imply that elevation and lowering of the LD content modulate the signal-induced release of Gce1- and CD73-containing adiposomes from adipocytes in negative and positive fashion respectively.

The impact of the reduced expression of Gce1, CD73, caveolin-1 and perilipin-A in the LD and increased release of Gce1 and CD73 into adiposomes on the coordinated inhibition of lipolysis and stimulation of esterification by GO,

glimepiride and palmitate, as reported recently (Müller *et al*., 2008c), was studied in rat adipocytes with lowered LD content in the course of long-term incubation with isoprenaline (Figure 9D and E). Under these conditions, short-term stimulation of lipolysis by isoprenaline, as measured by the release of glycerol and fatty acids into the incubation medium, was reduced by about 50% compared with control and isoprenaline plus orlistat-treated adipocytes (Figure 9D). This effect probably reflects the loss of intracellular TAG and LD rather than the desensitization of the β-adrenoceptor system. Significantly, the residual short-term lipolysis in the adipocytes with lowered LD content was not inhibited in the presence of GO, glimepiride and palmitate, which is in contrast to the 80–50% inhibition seen in cells with normal LD content (Figure 9D). After long-term incubation with isoprenaline, the palmitate- and glucose-driven esterification – measured as the incorporation of [$^{14}$C]palmitate and [$^{3}$H]glucose into TAG – was not significantly stimulated by GO, glimepiride and palmitate compared with basal, and remained below the 700–200% stimulation observed with the correspondingly induced control and isoprenaline plus orlistat-treated cells (Figure 9E). The relative ranking order of potency for both inhibition of the isoprenaline-stimulated lipolysis (Figure 9D) and stimulation of the palmitate- and glucose-driven esterification (Figure 9E) in control and isoprenaline plus orlistat-treated adipocytes with GO > glimepiride > palmitate was in agreement with previous findings (Müller *et al*., 2008c). Together, these results suggest that lowering of the LD content impairs the signal-induced inhibition of lipolysis, as well as stimulation of esterification in adipocytes. This presumably relies on the only transient and reduced expression of Gce1, CD73, caveolin-1 and perilipin-A in the LD (see Figure 9A, B and C) prior to their release into adiposomes, in adipocytes with reduced LD content, compared with cells with normal LD content. As a result, the cAMP degradation at the LD surface zone may be less efficient compared with signal-induced adipocytes with normal LD content.

*Role of LD biogenesis in the release of GPI-protein-containing adiposomes*

The data presented so far suggest that Gce1, CD73, caveolin-1 and perilipin-A are translocated from plasma membrane DIGs *via* LD to adiposomes in response to GO, glimepiride and palmitate. The involvement of DIGs and LD in the release of adiposomes was studied by its reconstitution in a cell-free system. This consisted of plasma membrane DIGs prepared from GO-treated photoaffinity-labelled adipocytes and LD from GO-treated unlabelled adipocytes. Upon incubation and subsequent sucrose density gradient centrifugation of the complete cell-free systems, the proteins from the recovered gradient fractions were analysed by SDS-PAGE/phosphorimaging or immunoblotting/lumiimaging (Figure 10). Photoaffinity-labelled Gce1 (Figure 10A) and immunoblotted caveolin-1 (Figure 10B) were detected in temperature (37 vs. 4°C)-, ATP- and time (60 vs. 5 min)-dependent fashion in a gradient fraction of high buoyant density (1.23 g·mL$^{-1}$), which is characteristic for adiposomes (Aoki *et al*., 2007; Müller *et al*., 2009), and in gradient fractions of low buoyant density (top – 1.02 g·mL$^{-1}$) containing LD (2nd panels). In contrast, the amounts of Gce1 and caveolin-1 remained unaltered in gradient fractions of medium buoyant density (1.10–1.14 g·mL$^{-1}$) containing DIGs. The time- and ATP-dependent upregulation of Gce1 and caveolin-1 in adiposomes and LD was significantly impaired when the DIGs and LD had been derived from control compared with GO-treated adipocytes (data not shown). Importantly, the recovery of Gce1 (Figure 10A) and caveolin-1 (Figure 10B) with LD and adiposomes was significantly diminished upon incubation of the cell-free system in the absence of LD (5th and 4th panels respectively). Thus, the *in vitro* reconstitution of the release of Gce1- and caveolin-1-containing adiposomes in a cell-free system critically depends on LD and ATP.

The apparent role of LD in the release of adiposomes from palmitate-, glimepiride- and GO-induced adipocytes may be based on their biogenesis rather than presence *per se*. Therefore, in order to discriminate between these two possibilities, TAG synthesis and LD biogenesis was blocked with the inhibitor of the acyl-CoA synthase, triacsin C (Iorio *et al*., 2003). Additionally, the assembly or fusion of primordial nascent LD into mature LD was impaired with various inhibitors ($AACOCF_3$, MAFP, PY-2, CPLAI) of the cytosolic group IVA phospholipase $A_2\alpha$ ($cPLA_2\alpha$). The requirement of $cPLA_2\alpha$ for LD biogenesis has previously been demonstrated for a number of non-adipocyte cell types and found to be unrelated to the generation of arachidonic acid, lipoprotein metabolism or TAG synthesis (Gubern *et al*., 2008). However, in that study, the role of the group VI phospholipase $A_2$ ($iPLA_2$) in LD biogenesis was not determined.

$AACOCF_3$, MAFP, PY-2, CPLAI and triacsin C significantly and concentration dependently reduced the amount of CD73-containing adiposomes released from rat adipocytes (which had been photoaffinity-labelled with [$^{14}$C]5′-FSBA and subsequently challenged with palmitate, glimepiride or GO) (Figure 11). For each inhibitor, the $IC_{50}$ values were comparable between the differently induced adipocytes and the maximal reductions of the adiposome release were highest for the palmitate versus glimepiride versus GO induction. The inhibitor of the $iPLA_2$, BEL, did not significantly affect the release of adiposomes from the induced adipocytes (Figure 11). These findings suggest that the GO-, glimepiride- and palmitate-induced release of GPI-protein-containing adiposomes requires biogenesis of LD, which involves both TAG synthesis and $cPLA_2\alpha$-dependent LD assembly.

## Discussion

*Involvement of DIGs and LD in the release of ADIP*

Almost all mammalian cell types so far investigated have been shown to release small membrane vesicles under certain physiological, pharmacological or stress conditions. The underlying mechanisms involve either shedding of the plasma membrane (microvesicles) or exocytosis of multivesicular bodies (exosomes) (Wolf, 1967; Poste and Nicolson, 1980; Heijnen *et al*., 1999; Stoorvogel *et al*., 2002; Thery *et al*., 2002; Freyssinet, 2003; Fevrier and Raposo, 2004; Cocucci *et al*., 2009). Primary and cultured rodent adipocytes also secrete both microvesicles and exosomes, collectively called adiposomes, in response to various stimuli, such as oxidative

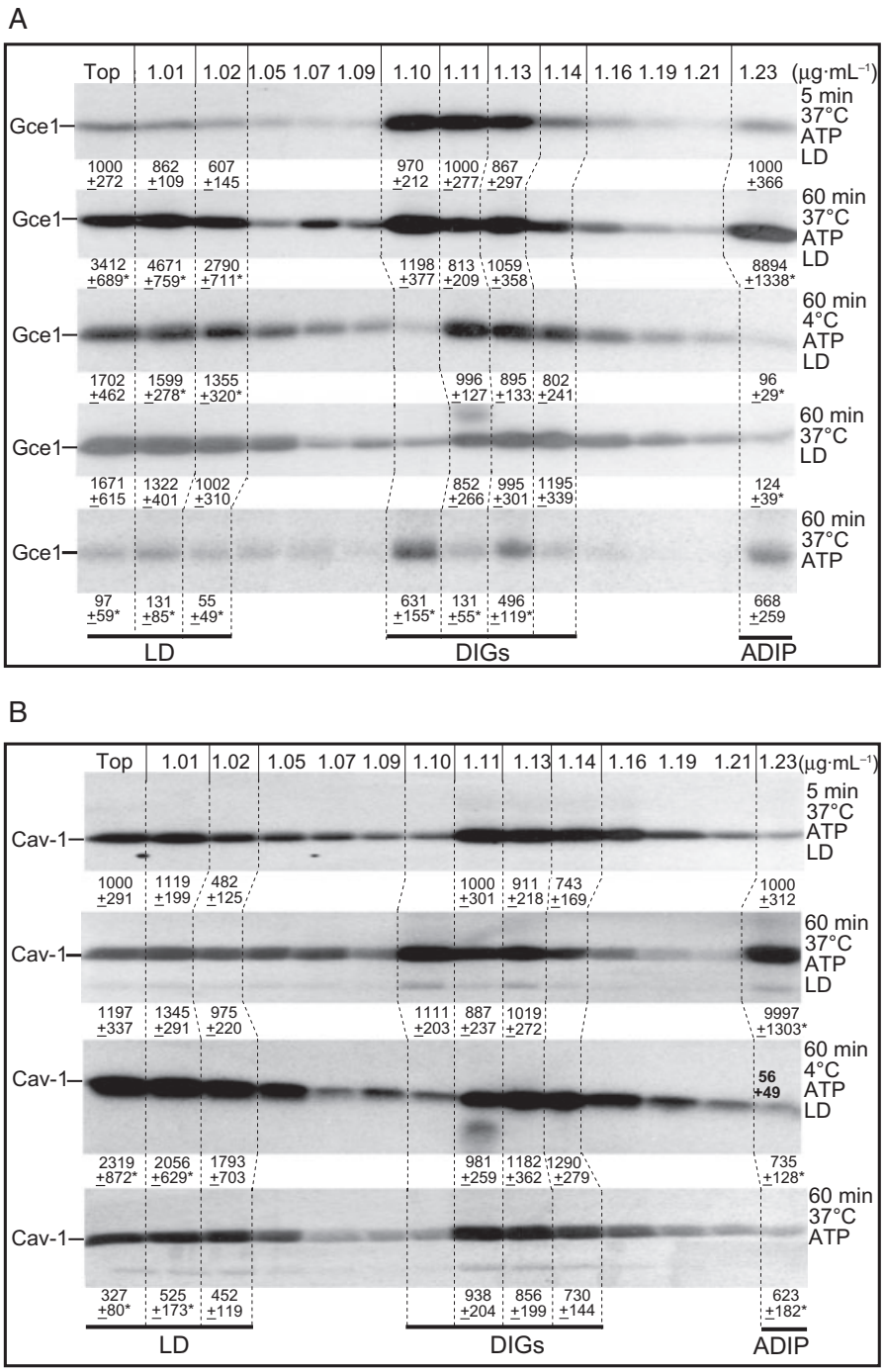


**Figure 10** Cell-free system for the release of adiposomes (ADIP) harboring Gce1 and caveolin-1. Plasma membrane DIGs (detergent-insoluble glycolipid-enriched plasma membrane microdomains) were prepared from glucose oxidase (GO)-stimulated rat adipocytes, which had been photoaffinity-labelled with 8-$N_3$-[$^{32}$P]cAMP. Lipid droplets (LD) were prepared from GO-stimulated unlabelled adipocytes. The DIGs were incubated (37 or 4°C, 5 or 60 min as indicated) without or with the LD in the absence or presence of an ATP-regenerating system (ATP). Thereafter, the incubation mixtures were subjected to sucrose gradient density centrifugation as described in Methods. Proteins were precipitated from the gradient fractions under denaturing conditions and then analysed for Gce1 by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphorimaging (A) or caveolin-1 (Cav-1) by immunoblotting and lumiimaging (B). Phosphor-/lumi-images from representative experiments are shown with quantitative evaluations (mean ± SD from three adipocyte preparations with incubations in triplicate) set at 1000 for the top (LD)-, 1.11 g·mL$^{-1}$ (DIGs)- and 1.23 g·mL$^{-1}$ (ADIP)-gradient fractions each from the 5 min incubation (upper panel) and marked with * for significant differences versus the corresponding gradient fractions from the 5 min incubation.

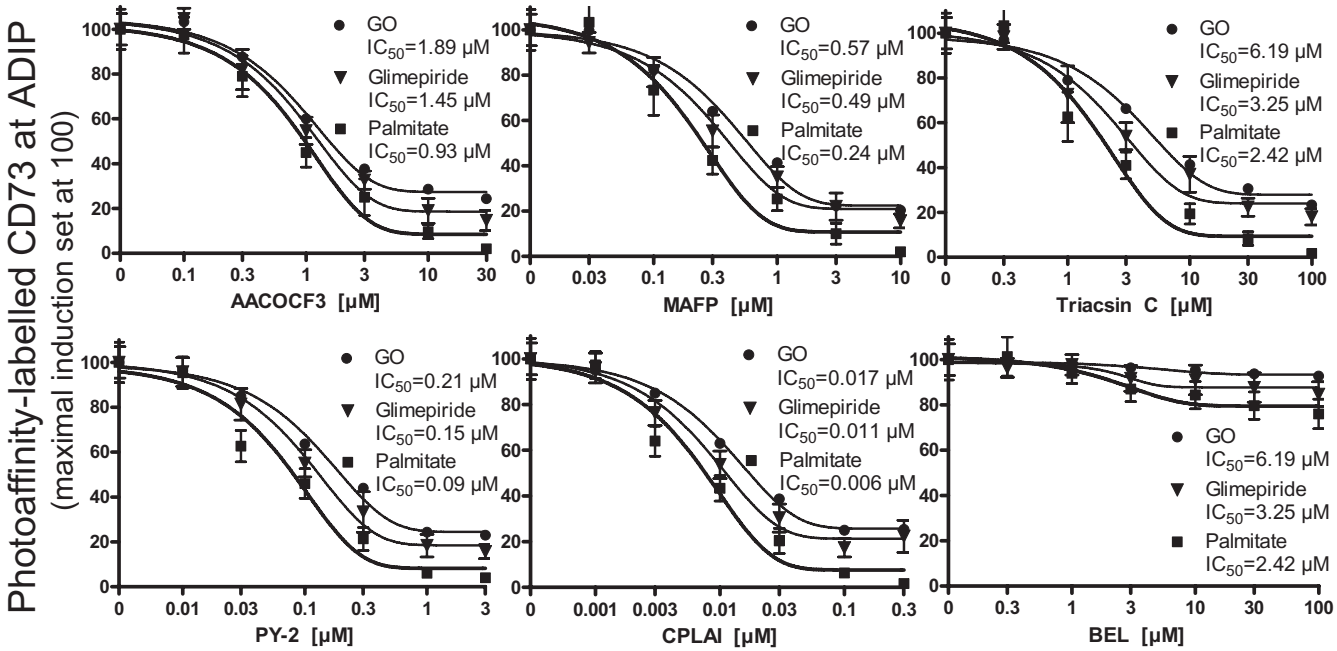


**Figure 11** Effect of inhibition of the lipid droplet (LD) biogenesis on the release of CD73 into adiposomes (ADIP). Isolated rat adipocytes were photoaffinity labelled with [$^{14}$C]5′-*p*-fluorosulphonylbenzoyladenosine, washed and then incubated (15 min, 37°C) in the absence or presence of increasing concentrations of triacsin C, methylarachidonyl fluorophosphate (MAFP), arachidonyl trifluoromethylketone ($AACOCF_3$), pyrrolidine-2 (PY-2), CPLAI ($cPLA_2$-specific inhibitor delivered by Calbiochem-Merck) and bromoenol lactone (BEL). After incubation (2 h, 37°C) of the adipocytes with GO (final conc. 500 mU·mL$^{-1}$), glimepiride (10 μM) or palmitate (1 mM), adiposomes were isolated from the incubation media and then extracted for proteins under denaturing conditions. Photoaffinity-labelled CD73 was analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis and phosphor imaging. Quantitative evaluations (mean ± SD from two adipocyte preparations with incubations in triplicate) are given with the maximal amount of photoaffinity-labelled CD73 in the absence of inhibitor set at 100 for each induction.

stress (Aoki *et al.*, 2007; Müller *et al.*, 2009), the anti-diabetic sulphonylurea drug glimepiride and excess of palmitate (Müller *et al.*, 2009). Adiposomes contain integral and peripheral plasma membrane and endosomal proteins, among them MFG-E8, caveolin-1 and perilipin-A. However, major and minor portions of caveolin-1 reside at DIGs and LD respectively. However, the opposite distribution is true for perilipin-A. Interestingly, caveolin-1, perilipin-A and two members of a different class of membrane proteins, namely the GPI proteins Gce1 and CD73 – with localization at both DIGs (major portion) and LD (minor portion) – have recently been identified as constituent components of rat adiposomes (Müller *et al.*, 2009).

The present results (Figure 1) extend these findings in that in response to specific signals, certain GPI proteins (Gce1), peripheral membrane proteins (perilipin-A) and integral membrane proteins (caveolin-1) become enriched in MFG-E8- and PC-containing adiposomes, which are constitutively released from adipocytes in the absence of those signals, albeit at a slightly lower level than in their presence. Thus, GPI-protein expression in adiposomes does not displace constitutive adiposome components and is not required for the constitutive adiposome release but it may drive the signal-induced release of perilipin-A-/caveolin-1-containing adiposomes. However, interference with Gce1 expression in adiposomes (as a consequence of inhibition of the GPI-PLC, see Figures 1 and 5) did not impair the release of perilipin-A into MFG-E8-containing adiposomes. This observation argues against GPI proteins operating as a trigger for the induced release of adiposomes. However, these conclusions have to be confirmed by proteomic analyses for the detection of putative compositional/structural differences between adiposomes released from adipocytes expressing or lacking Gce1/CD73 protein.

Principally, Gce1, CD73, caveolin-1 and perilipin-A may be released into adiposomes in the course of shedding and/or exocytosis. However, the present study provides experimental evidence for an alternative mechanism relying on their translocation from plasma membrane DIGs *via* LD to the adiposomes. This effect may be up-regulated by $H_2O_2$, glimepiride and palmitate: (i) pulse-chase photoaffinity and metabolic labelling, as well as long term-chase metabolic labelling of Gce1 and CD73 demonstrated that the two GPI proteins disappeared from the DIGs, then transiently appeared at the LD and were finally released into adiposomes (Figures 2 and 3). (ii) Release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes was prevented by interference with their translocation from plasma membrane DIGs to LD (Figure 5). (iii) Release of Gce1 and CD73 into adiposomes was prevented by disruption of the plasma membrane DIGs (Table 1, Figure 6). (iv) Release of Gce1 and CD73 into adiposomes was up-regulated by cholesterol depletion of LD (Figure 6). (v) Release of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes was down-regulated with increasing LD content (Figure 8). (vi) Release of Gce1 and CD73 into adiposomes was up-regulated with decreasing LD content (Figure 9A, B and C).

(vii) The decline in LD-associated and the simultaneous elevation in adiposome-associated Gce1 and CD73 in adipocytes with reduced LD content were accompanied by impaired lipolysis inhibition and esterification stimulation in response to $H_2O_2$, glimepiride and palmitate (Figure 9D and E). This coordinated control is known to depend on the expression of Gce1 and CD73 at the LD surface zone (Müller *et al.*, 2008a,c,d). (viii) Release of CD73 into adiposomes required the biogenesis of LD (Figure 11). (ix) The time-, temperature- and ATP-dependent release of Gce1- and caveolin-1-containing adiposomes in an adipocyte-derived cell-free system consisting of plasma membrane DIGs was strictly dependent on the presence of LD (Figure 10).

*Putative mechanisms for the release of DIGs- and LD-associated proteins into adiposomes*

The following findings argue for the involvement of classical vesicular transport in the release of adiposomes (i) Interference with endoplasmic reticulum-to-Golgi transport, using Golgi apparatus- or microtubule-disrupting reagents, blocked the expression of Gce1, CD73, caveolin-1 and perilipin-A in both LD and adiposomes (Figure 7A and B). (ii) The release of Gce1 into adiposomes depended on ATP and functional endocytosis (Figure 7C). (iii) The LD-, ATP- and temperature-dependent release of adiposomes in the cell-free system (Figure 10) depended upon intact membrane/vesicular structures, as it was completely inhibited by low concentrations of non-ionic detergents, which do not compromise the integrity of isolated adipocyte DIGs and LD (data not shown).

In addition to vesicular transport, both LD content and LD biogenesis affect the releasing efficacy of Gce1, CD73, caveolin-1 and perilipin-A into adiposomes in rat adipocytes. The underlying molecular basis may encompass the packaging of caveolin-1 and perilipin-A together with newly synthesized TAG into GPI-protein-containing nascent LD at the sites of LD biogenesis and/or the translocation of caveolin-1 and perilipin-A *via* pre-existing mature LD from plasma membrane DIGs to the sites of adiposome release. This hypothesis is supported by previous findings that the endoplasmic reticulum/endosomes and plasma membrane DIGs, the sites of exosome and microvesicle biogenesis, have also been implicated as major and minor sites, respectively, of LD biogenesis in mammalian adipocytes (Öst *et al.*, 2005; Robenek *et al.*, 2006; Örtegren *et al.*, 2007; Ducharme and Bickel 2008).

Recently, inhibition of LD biogenesis by specific blockade of the $cPLA_2\alpha$ has been observed in a variety of non-adipose cell lines (Gubern *et al.*, 2008). Thus, it is conceivable that the induced release of CD73-containing adiposomes by inhibition of the $cPLA_2\alpha$ in primary rat adipocytes, as revealed in the present study (Figure 11), relies on the inhibition of the $cPLA_2\alpha$-dependent assembly of GPI proteins, caveolin-1 and perilipin-A into LD at the endoplasmic reticulum/endosomes and/or plasma membrane DIGs. The latter site may be specific for adipocytes since accumulation of TAG at the plasma membrane in the course of down-regulated LD biogenesis has not been observed for non-adipose cell lines (Gubern *et al.*, 2008).

Although $cPLA_2\alpha$ predominantly resides in the cytosol and at cytoplasmic LD in the basal state (Yu *et al.*, 2005), there is good experimental evidence for its $Ca^{2+}$- and phosphorylation-dependent association with phospholipid substrates at the endoplasmic reticulum/endosomes and plasma membranes in the induced state (Gosh *et al.*, 2006). Together, these findings are compatible with the involvement of sites of LD biogenesis in the mechanism of adiposome release. This would easily explain the sharing of a number of protein components, such as Gce1, CD73, caveolin-1 and perilipin-A, and of free cholesterol between adipocyte LD and adiposomes (Müller *et al.*, 2008a; 2009).

However, the coupling of LD biogenesis and adiposomes release *via* shedding and/or exocytosis would lead to vesicles containing TAG and cholesteryl ester in their core covered by a phospholipid monolayer with the protein components (derived from the LD) surrounded by a phospholipid bilayer (derived from the plasma membranes and/or endosomes). However, this 'LD-in-vesicle' structure is very unlikely for adiposomes, since the presence of TAG and cholesterylester has not been reported so far. Moreover, Gce1 and CD73 seem to be exposed at the adiposome surface rather than covered by a membrane bilayer on the basis of the following findings: (i) Gce1 and CD73 were accessible to photoaffinity labelling by membrane-impermeable photoprobes using intact adiposomes (Figure 2); (ii) photoaffinity-labelled Gce1 and CD73 were rapidly degraded by exogenous V8 protease added to intact adiposomes (data not shown); (iii) photoaffinity-labelled Gce1 and CD73 were rapidly released from intact adiposomes upon degradation of their GPI anchors by exogenously added (G)PI-specific PLC (Müller *et al.*, 2005; 2008c,e); (iv) Gce1 and CD73 can be assayed for their enzymic activities by the addition of membrane-impermeable substrates to intact adiposomes (Figure 9B); and (v) the enzymic activities of Gce1 and CD73 were not cryptic, that is, fail to become elevated upon detergent solubilization of intact adiposomes (data not shown).

Thus the combined experimental evidence argues against vesicularization of LD in course of shedding and/or exocytosis as mechanism for the release of GPI-protein-, caveolin-1- and perilipin-A-containing adiposomes.

Clearly, the apparent differences in the structure and topology of plasma membrane DIGs, LD and adiposomes have to be considered for putative models of adiposome release. Their constituent proteins, Gce1 and CD73, are located at the exoplasmic leaflet of the adiposome and plasma membrane bilayers, but at the cytoplasmic leaflet of the LD monolayer. Spontaneous or protein (channel)-mediated transmembrane movement of GPI proteins between membrane leaflets has not been reported so far. The lipid-based 'escape hatch' model recently proposed for the removal of defective membrane proteins from the endoplasmic reticulum (Ploegh, 2007) can be adapted for the translocation of GPI proteins from plasma membrane DIGs *via* LD to adiposomes. It involves the (transient) formation of bicellar structures, created by fusion of the exoplasmic and cytoplasmic leaflets of the relevant membrane, into which Gce1, CD73, caveolin-1 and perilipin-A would be embedded. A 'wrinkled' surface could create ridges with local bilayer properties compatible with conventional anchorage of GPI proteins and transmembrane/peripheral membrane proteins. Both lipid composition and membrane curvature may prepare the excision of GPI protein-containing nascent LD from relevant

membranes by bicelle formation, which subsequently mature by accumulation of TAG.

The newly formed Gce1-, CD73-, caveolin-1- and perilipin-A-containing LD may mix and distribute within the pre-existing large central LD, possibly without undergoing fusion. This putative floating of 'islets' of GPI-protein-containing LD within the 'sea' of pre-existing LD could explain the inverse correlation between LD content and the efficacy of adiposome release (Figures 8 and 9). Upon segregation of the TAG/cholesterol into the pre-existing LD, the subsequent translocation of Gce1, CD73, caveolin-1 and perilipin-A to the sites of their release into adiposomes by exocytosis and/or shedding may engage the same bicelle-based mechanism operating at endosomal membranes and/or plasma membranes, respectively, albeit in reverse order. Apparently, one or more of these steps depends on vesicular transport (Figure 7) and is controlled by physiological and pharmacological signals, such as $H_2O_2$, palmitate and glimepiride.

There is a precedence for a molecular mechanism regulating the translocation of proteins from plasma membrane DIGs to LD. During adipocyte differentiation caveolin-1 – commonly assumed to be important for the homeostasis of the LD cholesterol pool (Dugail *et al*., 2003) – is translocated from the plasma membrane to the LD in response to cholesterol, fatty acids and heavy lipid loading (Pol *et al*., 2005; Le Lay *et al*., 2006; Blouin *et al*., 2008). The induced translocation of caveolin to and the coating of the mature LD with caveolin has recently been shown to involve activation of Src family kinases and presumably phosphorylation of caveolin (Le Lay *et al*., 2006). Interestingly, the Src family kinase, c-Lyn, is known to redistribute between high and low cholesterol-containing plasma membrane DIGs and become concomitantly activated in response to glimepiride in rat adipocytes (Müller *et al*., 2001; 2002; 2005). Together, these findings are suggestive of a role of the tyrosine phosphorylation of caveolin as an initial trigger for its translocation from DIGs to LD. It will be interesting to study the role of Src family kinases in the translocation of caveolin-1, Gce1, CD73 and perilipin-A from plasma membrane DIGs to LD.

*Putative physiological role of the involvement of LD in the release of ADIP*

During translocation from plasma membrane DIGs to LD Gce1, CD73, caveolin-1 and perilipin-A are apparently accompanied by cholesterol. This is suggested by similar time courses of their redistribution during pulse-/long-term chase metabolic labelling (Figures 3 and 4). Moreover, similarities exist in the inhibition of the GO-induced translocation of Gce1 and CD73 from DIGs to LD upon disruption of the DIGs by cholesterol depletion before their metabolic labelling (Figure 6). So far, plasma membranes and LD have been identified as the major compartments of cholesterol accumulation in adipocytes. Numerous studies have highlighted a strong correlation between adipocyte cholesterol content and LD content (Dugail *et al*., 2003), that is, the higher the LD content, the greater the need for cholesterol transport from plasma membranes to LD. An exciting possibility is that the translocation of cholesterol in concert with Gce1, CD73, caveolin-1 and perilipin-A from plasma membrane DIGs to LD might serve as intracellular signal for the increase in LD content, that is, LD size and number, during adipocyte differentiation and enlargement. The mature large adipocytes may then release adiposomes containing Gce1, CD73, caveolin-1 and perilipin-A, which act upon neighbouring preadipocytes and young small adipocytes. Thus, the released adiposomes may prepare the recipient cells for excessive TAG storage and LD biogenesis. Compatible with this hypothesis is the present finding of strict dependence of the amount of adiposomes released in both constitutive and regulated fashion from the cell size, with large adipocytes being considerably more efficient than small ones (Figure 1). Intercellular signalling functions have previously been suggested for microvesicles and exosomes from non-adipose cells and tissues (Wolf, 1967; Poste and Nicolson, 1980; Heijnen *et al*., 1999; Denzer *et al*., 2000; Fritzsching *et al*., 2002; Stoorvogel *et al*., 2002; Thery *et al*., 2002; Freyssinet, 2003; Fevrier and Raposo, 2004; Al-Nedawi *et al*., 2008).

Surprisingly, the accumulation of cholesterol at LD apparently impairs the signal-induced release of Gce1 and CD73 from LD into adiposomes, as revealed by its upregulation upon cholesterol depletion of the LD harbouring metabolically labelled Gce1 and CD73 (Figure 6). These findings suggest a dual and seemingly counterproductive role of cholesterol in the control of the signal-induced translocation of GPI proteins from DIGs to LD (positive) and release into adiposomes (negative). Possibly, LD with Gce1 and CD73 (accumulated in concert with cholesterol during TAG synthesis in large adipocytes) operate as storage compartments and sources for the subsequent rapid release of these GPI proteins into adiposomes in response to extracellular signals. In adipocytes with reduced LD content (in consequence of long term lipolytic treatment), the inhibition of lipolysis and stimulation of esterification by $H_2O_2$, glimepiride and palmitate are considerably impaired (Figure 9). This 'feed-forward' mechanism may result in ongoing lipolytic degradation and consequently in complete loss of LD from these adipocytes, possibly as a prerequisite for their subsequent degeneration.

## Conflict of interest

The authors are employees of Sanofi-Aventis Pharma.

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