Insulin stimulates the release of the glycosyl phosphatidylinositol-anchored membrane dipeptidase from 3T3-L1 adipocytes through the action of a phospholipase C

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Membrane dipeptidase (MDP; EC 3.4.13.19) enzymic activity that was inhibited by cilastatin has been detected on the surface of 3T3-L1 cells. On differentiation of the cells from fibroblasts to adipocytes the activity of MDP increased 12-fold. Immunoelectrophoretic blot analysis indicated that on adipogenesis the increase in the amount of MDP preceded the appearance of GLUT-4. MDP on 3T3-L1 adipocytes was anchored in the bilayer by a glycosyl phosphatidylinositol (GPI) moiety as evidenced by its release into the medium in a hydrophilic form on treatment of the cells with bacterial phosphatidylinositol-specific

phospholipase C and the appearance of the inositol 1,2-cyclic monophosphate cross-reacting determinant. Incubation of 3T3-L1 adipocytes with either insulin or the sulphonylurea glimepiride led to a rapid concentration- and time-dependent release of MDP from the cell surface. The hydrophilic form of MDP released from the cells on stimulation with insulin was recognized by antibodies against the inositol 1,2-cyclic monophosphate cross-reacting determinant, indicating that it had been generated by cleavage of its GPI anchor through the action of a phospholipase C.

INTRODUCTION

Glycosyl phosphatidylinositol (GPI) anchors are found on a diverse range of eukaryotic cell surface proteins (reviewed in [1]). Although a GPI anchor can be considered primarily as an alternative to a hydrophobic transmembrane polypeptide anchor, several other functions have been proposed for GPI anchors. These include roles in intracellular sorting [2], in the endocytic process of potocytosis [3] and in transmembrane signalling [4]. A GPI anchor might also allow a protein to associate in membrane microdomains [5], to be more laterally mobile in the bilayer [6], and to be selectively released from the bilayer by phospholipases.

A mammalian GPI-specific phospholipase D has been isolated, cloned and sequenced, and shown to exist predominantly as a soluble protein in serum [7–9], although a cell-associated form has also been described [10]. Soluble phospholipase D might be involved in the release of the membrane-bound form of alkaline phosphatase from the sinusoidal surface of hepatocytes [11], and the cell-associated form seems to be involved in the release of decay-accelerating factor from HeLa cells and heparan sulphate proteoglycan from bone marrow stromal cells [10]. Several studies have identified putative forms of mammalian GPI-specific phospholipase C on the basis of the release of diacylglycerol from GPI anchors [12–14]. However, a later report revealed that such activities might be due to the combined action of a phospholipase D releasing phosphatidic acid from GPI anchors and its subsequent dephosphorylation by a phosphatase to form diacylglycerol [15]. Despite this, there is strong evidence indicating that such GPI-specific phospholipase C forms exist. For example, using anti-(cross-reacting determinant) (CRD) antibodies that selectively recognize the inositol 1,2-cyclic monophosphate epitope formed on cleavage of a GPI anchor by a phospholipase C [16,17], we showed that the soluble form of the GPI-anchored 5'-nucleotidase in bovine cerebral cortex and the electric organ of the electric ray was derived from the membrane-bound form through the action of a phospholipase C [18].

Although insulin is probably the most extensively studied hormone, the molecular events that result in some of its cellular actions are poorly understood [19]. Some of the cellular actions of insulin have been proposed to be mediated by the novel second messengers, the inositol phosphoglycan (IPG) mediators (reviewed in [20–22]). In addition to being chemically and structurally related to the GPI anchors on cell surface proteins, IPGs are also immunologically similar. For example, antibodies against the glycan core of GPI anchors can selectively block some of the metabolic actions of insulin on intact cells [23], and our anti-CRD antisera recognize both the synthetic insulin-mimetic disaccharide, glucosaminyl-1,6-inositol-1,2-cyclic monophosphate [17] and a natural IPG mediator isolated from human plasma [24]. The observation that in a cell line defective in the synthesis of GPI anchors, insulin is unable to stimulate glycogen synthesis suggests that GPI structures are critically involved in some of the cellular actions of insulin [25]. However, it is not entirely clear whether IPGs are derived from the cleavage of GPI anchors on proteins or from related free GPIs [26], and whether this is through the action of a phospholipase C or D. As a first step towards elucidating the relationship between soluble IPG mediators and GPI anchors on cell surface proteins we have investigated the insulin stimulated release of GPI-anchored proteins from 3T3-L1 adipocytes. In the present study we show for the first time that the well-characterized mammalian GPI-anchored protein, membrane dipeptidase (MDP; EC 3.4.13.19) is present on the surface of 3T3-L1 cells and that its expression increases in line with adipogenesis. We also show that insulin stimulates the release of MDP from the surface of 3T3-L1 cells through the action of a phospholipase C.

EXPERIMENTAL

Materials

Cilastatin was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Phosphatidylinositol-speci-

Abbreviations used: CRD, cross-reacting determinant; DMEM, Dulbecco's modified Eagle's medium; GPI, glycosyl phosphatidylinositol; IPG, inositol phosphoglycan; KRH, Krebs–Ringer Hepes buffer; MDP, membrane dipeptidase; PI-PLC, phosphatidylinositol-specific phospholipase C.

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fic phospholipase C (PI-PLC) from *Bacillus thuringiensis* was a gift from Dr. M. G. Low (Columbia University, New York, NY, U.S.A.). The units of PI-PLC activity are $\mu\text{mol}/\text{min}$. *Trypanosoma brucei* variant surface glycoprotein was a gift from Professor M. A. J. Ferguson (University of Dundee, Dundee, U.K.). Glimepiride was a gift from Hoechst Roussel (Swindon, Wilts., U.K.). Anti-GLUT4 antiserum was a gift from Dr. S. A. Baldwin (University of Leeds, Leeds, U.K.). Dulbecco's modified Eagle's medium (DMEM), pig insulin, pig glucagon, human platelet-derived growth factor, methylisobutylxanthine, dexamethasone, foetal calf serum and Gly-D-Phe were from Sigma Chemical Co. (Poole, Dorset, U.K.). Newborn calf serum was from Gibco (Paisley, Strathclyde, U.K.). MDP was purified from pig kidney cortex after solubilization with PI-PLC by affinity chromatography on cilastatin-Sepharose [27]. Protein was determined by the bicinchoninic acid method of Smith et al. [28] modified for use in 96-well microtitre plates [29] with BSA as standard.

Cell culture

3T3-L1 fibroblasts (American culture collection) were seeded at a density of 2×10^4 cells/ml, grown in DMEM containing 5% (v/v) newborn calf serum, 50 i.u./ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate, and maintained in a humidified atmosphere of air/ CO_2 (9:1) at 37 °C; 3 days after the cells had achieved confluence, differentiation was induced with DMEM containing 2 mM glutamine, 10% (v/v) foetal calf serum, 1 $\mu\text{g}/\text{ml}$ insulin, 0.25 μM dexamethasone, 0.5 mM methyl isobutylxanthine, 50 i.u./ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate. After a further 3 days the cells were incubated in the same medium without methyl isobutylxanthine and dexamethasone for 3 days more. Subsequently the cells were maintained in DMEM containing 10% (v/v) foetal calf serum and antibiotics. The medium was changed at intervals of 2–3 days. Cells were used 6–9 days after the induction of differentiation. Approx. 80–90% of these cells expressed the adipocyte phenotype.

Membrane preparation

Cells were scraped from the flasks into PBS [20 mM $\text{Na}_2\text{HPO}_4/2$ mM $\text{NaH}_2\text{PO}_4/150$ mM NaCl (pH 7.4)] and centrifuged at 100 g for 10 min. The cells were disrupted in a nitrogen bomb at 800 lb/in² (5520 kPa) for 10 min. The suspension was centrifuged at 1000 g for 10 min; the resulting supernatant was centrifuged at 100000 g for 90 min. The resulting membrane pellet was resuspended in 10 mM Hepes/NaOH, pH 7.4.

Responsiveness of 3T3 adipocytes to insulin

The responsiveness of 3T3 adipocytes to insulin was assessed by measuring deoxyglucose uptake. 3T3 adipocytes were incubated in serum-free medium overnight at 37 °C, washed three times in Krebs–Ringer Hepes buffer, pH 7.4 (KRH; 128 mM NaCl/4.7 mM KCl/1.25 mM $\text{CaCl}_2/1.25$ mM $\text{MgSO}_4/10$ mM Hepes) and incubated with 1 μM insulin for 10 min at 37 °C. 2-[³H]Deoxyglucose (5 mCi/mol) was added to the cells to a final concentration of 0.2 mM. The uptake was terminated by aspirating the medium and washing the cells with ice-cold PBS. The cells were permeabilized with 1% (v/v) Triton X-100; a 250 μl sample was removed from each flask for scintillation counting.

Incubation of adipocytes with insulin, glimepiride or PI-PLC

Insulin was dissolved at a final concentration of 1 mg/ml in 0.01 M HCl. Glimepiride was dissolved in 25 mM Hepes/KOH,

pH 7.4, to give a 2 mM stock solution made fresh daily. The cells were incubated in serum-free medium for 2 h, then washed three times in KRH containing 5 mM glucose. The 3T3-L1 adipocytes were incubated at 37 °C for various periods with the indicated concentrations of PI-PLC, insulin or glimepiride in KRH. The medium was then removed, centrifuged to remove any free cells and concentrated 10-fold with Vivascience 15 concentrators (10 kDa cut off) before being assayed for protein and enzyme activity.

MDP assay

MDP enzymic activity was assayed with Gly-D-Phe (1 mM) as substrate in 0.1 M Tris/HCl, pH 8.0. Reactions were terminated by heating at 100 °C for 4 min, and the product D-Phe was separated from the substrate and quantified by reverse-phase HPLC as described previously [30]. For the determination of cell surface MDP activity, cells were incubated in serum-free medium for 2 h, washed three times with KRH and then incubated with 1 mM Gly-D-Phe for 1 h in KRH. The medium was then removed and centrifuged for 10 min at 12000 g ; the amount of D-Phe product was quantified by reverse-phase HPLC.

SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17% (w/v) polyacrylamide gradient as described previously [31]. Immunoelectrophoretic blot analysis was performed with Immobilon P PVDF membranes as described previously [32]. A polyclonal antiserum against pig kidney MDP was generated in rabbits [27]. Anti-CRD antibodies were isolated from the bulk of the anti-MDP antiserum by fractionation on a column of an immobilized soluble form of the trypanosome variant surface glycoprotein [16,17]. The detection of bound antibody was performed with a peroxidase-conjugated secondary antibody in conjunction with the chemiluminescence detection method (ECL kit, Amersham). Molecular mass standards were run in parallel and were revealed by staining with Coomassie Brilliant Blue. The CRD was selectively destroyed by incubation of the protein sample in 1 M HCl for 30 min at room temperature before electrophoresis [16].

Precipitation of MDP with cilastatin-Sepharose

Cilastatin-Sepharose was prepared as described previously [27]. Medium from 3T3-L1 cells was incubated with cilastatin-Sepharose for 18 h at 4 °C in 50 mM Tris/HCl (pH 7.6)/0.5 M NaCl. After extensive washing with the same buffer, bound material was eluted from the cilastatin-Sepharose by boiling for 4 min in the presence of SDS dissociation buffer. The samples were then subjected to SDS/PAGE and immunoelectrophoretic blot analysis as described above.

Temperature-induced phase separation in Triton X-114

Triton X-114 was precondensed before use [33]. To each sample was added an equal volume of 10 mM Tris/HCl (pH 7.4)/0.15 M NaCl/2% (v/v) Triton X-114. The samples were incubated on ice for 5 min, followed by incubation at 30 °C for 3 min. After centrifugation at 3000 g for 3 min, the detergent-poor phase was removed to a clean tube and the detergent-rich phase made up to the same volume as the detergent-poor phase with 10 mM Tris/HCl (pH 7.4)/0.15 M NaCl before determination of MDP enzymic activity.

Figure 1 Increase in cell surface MDP activity on 3T3-L1 cells in line with adipogenesis

Cell surface MDP activity was determined by adding Gly-D-Phe (1 mM final concentration) to the culture medium. After 1 h the medium was removed and the breakdown of Gly-D-Phe assessed by reverse-phase HPLC as described in the Experimental section. No Gly-D-Phe hydrolysis was detected at any stage of differentiation in the presence of the specific inhibitor of MDP, cilastatin. Results are means $\pm$ S.E.M. for triplicate determinations.

Figure 2 Immunoelectrophoretic blot of membrane preparations from 3T3 cells with anti-MDP and anti-GLUT4 antisera

Membranes were prepared from 3T3-L1 cells 3 days after plating out and at 3 day intervals thereafter as described in the Experimental section. Samples were subjected to electrophoresis under reducing conditions and subjected to immunoelectrophoretic blot analysis with either an anti-MDP antiserum (upper panel) or an anti-GLUT4 antiserum (lower panel). The amount of protein applied to each lane was 25 μ g.

RESULTS

MDP is expressed on the surface of 3T3-L1 cells

Membranes were isolated from both 3T3-L1 fibroblasts (undifferentiated) and adipocytes (differentiated) as described in the Experimental section and assayed for the presence of MDP. The responsiveness of the adipocytes to insulin was confirmed by monitoring the uptake of 2-[3 H]deoxyglucose (see the Experimental section). MDP enzymic activity that was inhibited by the specific inhibitor cilastatin was detected in membrane preparations from both fibroblasts (2.0 ± 0.1 nmol of D-Phe/min per mg of protein; $n = 3$, means $\pm$ S.E.M.) and adipocytes (24.2 ± 1.0 nmol of D-Phe/min per mg of protein; $n = 3$, means $\pm$ S.E.M.). The increase in cell surface activity of MDP on 3T3-L1 cells on differentiation was examined in more detail (Figure 1). Although MDP enzymic activity was detected on the surface of undifferentiated cells, the level of activity increased markedly

Figure 3 PI-PLC releases MDP from the surface of 3T3-L1 cells

3T3-L1 adipocytes were incubated in the presence of 0.1 m-unit/ml *B. thuringiensis* PI-PLC. At the indicated times, the culture medium was withdrawn and concentrated and MDP enzymic activity was determined as described in the Experimental section. No release of MDP into the culture medium was observed from untreated cells. Results are means $\pm$ S.E.M. for triplicate determinations.

Table 1 Phase separation in Triton X-114 of MDP released from 3T3-L1 cells by either bacterial PI-PLC or insulin

Membranes from 3T3-L1 cells after incubation in the absence or presence of 0.1 unit of *B. thuringiensis* PI-PLC for 1 h at 37 $^{\circ}$ C, or medium from either insulin-stimulated or PI-PLC-treated cells, were subjected to phase separation in Triton X-114 as described in the Experimental section. The resulting detergent-rich and detergent-poor phases were assayed for MDP activity, and the activity recovered in the detergent-poor phase is expressed as a percentage of the total activity in both phases. Results are means of duplicate determinations.

Sample	Activity in detergent-poor phase (% of total activity)
3T3-L1 adipocyte membranes	5.3
3T3-L1 adipocyte membranes + PI-PLC	87.8
Medium from PI-PLC-treated 3T3-L1 cells	93.4
Medium from insulin-stimulated 3T3-L1 cells	89.6

with the onset of differentiation, increasing in line with adipogenesis. The appearance of MDP on differentiation was compared with that of the GLUT-4 transporter protein (Figure 2). Immunoelectrophoretic blot analysis of membranes prepared from 3T3-L1 cells at different stages of differentiation with an anti-MDP antibody indicated that MDP protein of molecular mass 45 kDa increased in line with the increase in cell surface enzymic activity. Interestingly the appearance of MDP seemed to precede the appearance of GLUT-4 by approx. 3 days (Figure 2).

MDP is GPI-anchored on 3T3-L1 adipocytes

The presence of a GPI anchor on MDP expressed on the surface of 3T3-L1 adipocytes was assessed by incubation with *B. thuringiensis* PI-PLC (Figure 3). MDP was released into the cell medium in a time-dependent manner on incubation of the cells with PI-PLC, with the released form partitioning predominantly into the detergent-poor phase on phase separation in Triton X-114 (Table 1). Similarly, on incubation of adipocyte membranes

Figure 4 Immunoelectrophoretic blot of insulin- and PI-PLC-released MDP with an anti-CRD antiserum

MDP was isolated from the media of either insulin- or PI-PLC-treated 3T3-L1 cells with cilastatin–Sepharose, as described in the Experimental section, before immunoelectrophoretic blot analysis with an anti-MDP antiserum (a) or an anti-CRD antiserum (b). Lane 1, PI-PLC released, affinity-purified pig kidney MDP treated with 1 M HCl before electrophoresis (0.5 μ g of protein); lane 2, PI-PLC released, affinity-purified pig kidney MDP (0.5 μ g of protein); lane 3, MDP released from 3T3-L1 cells with PI-PLC (0.3 μ g of protein); lane 4, MDP released from 3T3-L1 cells with insulin (0.3 μ g of protein); lane 5, trypanosome variant surface glycoprotein (0.5 μ g of protein).

with PI-PLC, MDP was converted from an amphipathic form into a hydrophilic form as assessed by phase separation in Triton X-114 (Table 1). The presence of a GPI anchor on MDP was confirmed by immunoelectrophoretic blot analysis with an anti-CRD antiserum (Figure 4). MDP released from the 3T3-L1 cells by bacterial PI-PLC was isolated from the media by precipitation with cilastatin–Sepharose as described in the Experimental section. The released MDP protein was recognized by both an anti-MDP antiserum (Figure 4a, lane 3) and an anti-CRD antiserum (Figure 4b, lane 3). The cross-reactivity with the anti-CRD antiserum indicates the presence of the inositol 1,2-cyclic monophosphate epitope that is generated on cleavage of the GPI anchor by phospholipase C [16,17]. The specificity of the anti-CRD antiserum was shown by its recognition of the soluble form of the trypanosome variant surface glycoprotein (Figure 4b, lane 5), and by the loss of recognition of MDP pretreated with 1 M HCl (Figure 4b, lane 1).

Insulin stimulates the release of MDP from 3T3-L1 adipocytes by a phospholipase C

Incubation of 3T3-L1 adipocytes with insulin caused a time- and concentration-dependent release of MDP from the surface of the cells, as assessed by monitoring for the appearance of MDP enzymic activity in the media (Figures 5a and 5b). Maximal release was observed with 10–100 nM insulin, with half-maximal release observed at an insulin concentration of 1.2 nM (Figure 5a). The maximal release observed with insulin was 45% of the maximal release observed with PI-PLC. The release of MDP on stimulation with insulin was rapid, with maximal release occurring after 20 min and no further release apparent up to 1 h (Figure 5b). In contrast with insulin, when 3T3-L1 adipocytes were incubated with either 100 nM glucagon or 1 nM platelet-derived growth factor for 15 min, no release of MDP into the medium above background levels was observed. MDP released from the cells on stimulation with insulin was precipitated from the media with cilastatin–Sepharose and subjected to immunoelectrophoretic blot analysis with either an anti-MDP antiserum or an anti-CRD antiserum. MDP released on stimulation with insulin migrated with an identical apparent molecular mass to

Figure 5 Insulin induces the release of MDP from 3T3-L1 cells

(a) 3T3-L1 adipocytes were incubated in the presence of the indicated concentration of insulin. After 15 min the culture medium was withdrawn and concentrated and MDP activity was determined as described in the Experimental section. (b) 3T3-L1 adipocytes were incubated in the presence of 100 nM insulin. At the indicated times, the culture medium was withdrawn and concentrated and MDP activity was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate determinations.

the PI-PLC-released form (Figure 4a, lanes 3 and 4) and was hydrophilic as assessed by phase separation in Triton X-114 (Table 1). In addition, the insulin-released MDP was recognized by the anti-CRD antiserum (Figure 4b, lane 4), indicating that its GPI anchor had been cleaved by a phospholipase C generating the inositol 1,2-cyclic monophosphate CRD epitope.

The sulphonylurea glimepiride stimulates the release of MDP from 3T3-L1 adipocytes

Incubation of 3T3-L1 adipocytes with glimepiride caused a time- and concentration-dependent release of MDP from the cell surface (Figure 6). Maximal release was observed with 5 mM glimepiride, with half-maximal release observed at a glimepiride concentration of 16 μ M (Figure 6a). The maximal release of

Figure 6 Glimepiride induces the release of MDP from 3T3-L1 cells

(a) 3T3-L1 adipocytes were incubated in the presence of the indicated concentration of glimepiride. After 30 min the culture medium was withdrawn and concentrated and MDP activity was determined as described in the Experimental section. (b) 3T3-L1 adipocytes were incubated in the presence of 50 μ M glimepiride. At the indicated times, the culture medium was withdrawn and concentrated and MDP activity was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate determinations.

MDP observed with glimepiride was 30 % of the maximal release observed with PI-PLC. The release of MDP on stimulation of the cells with glimepiride was rapid, with maximal release occurring after 20 min and no further release apparent up to 1 h (Figure 6b).

3T3-L1 cells were incubated in the presence of either insulin or glimepiride, the medium was removed and the cells were then further incubated in the presence of PI-PLC, insulin or glimepiride (Table 2). After an initial treatment with insulin, subsequent treatment of the cells with PI-PLC caused a significant release of MDP into the medium, whereas subsequent incubation without the addition of PI-PLC resulted in no further release of MDP. Negligible MDP was released from the cell surface after further treatment of the insulin-stimulated cells with either insulin or glimepiride. Essentially identical results were obtained if the

cells were first pretreated with glimepiride. Thus although after insulin or glimepiride treatment there is still a large amount of MDP on the cell surface that is susceptible to release by PI-PLC, there seems to be a significant decrease in the insulin/glimepiride-releasable pool of MDP.

DISCUSSION

MDP is a zinc metalloenzyme that is located on the brush-border membrane of the kidney and the intestine, and is also present in the lungs and pancreas [34,35]. However, the presence of the enzyme on adipocytes has not been previously reported. The observation that MDP cell surface activity increases markedly on differentiation of the 3T3-L1 cells from fibroblasts to adipocytes suggests that the enzyme might have a critical role in the development or maintenance of the differentiated phenotype. Interestingly, growth of cells in the presence of the MDP inhibitor cilastatin in the media leads to an increase in the rate of cell growth and differentiation (S. Movahedi and N. M. Hooper, unpublished work). One of the possible physiological roles of MDP on the surface of adipocytes is the metabolism of peptide hormone signals, and in this context it is interesting to note that another ectopeptidase, the GLUT4 vesicle-associated aminopeptidase (gp160/vp165), has also recently been identified in adipocytes [36–38]. However, it should be borne in mind that, as its name implies, the action of MDP is restricted to dipeptides and related structures such as leukotriene D₄ [39].

MDP is one of the best characterized mammalian GPI-anchored proteins (reviewed in [34]). Pig MDP was the first mammalian peptidase that was found to be GPI-anchored [30]; subsequently MDP has been shown to be GPI-anchored in several species, including humans [40] and mice [41]. The GPI anchor on both pig and human MDP has been extensively characterized in terms of its hydrolysis by bacterial PI-PLC and a plasma phospholipase D [27,42,43], and the complete structure of the GPI anchor on pig MDP has been determined, as has the glycan core structure of human MDP, representing the only inter-species comparison of the GPI anchor on the same protein [44]. In the present study we provide unequivocal evidence that MDP on the surface of 3T3-L1 adipocytes is also GPI-anchored, as demonstrated by its susceptibility to release by bacterial PI-PLC and the subsequent generation of the inositol 1,2-cyclic monophosphate CRD epitope.

Insulin has been shown to stimulate the release of several mammalian GPI-anchored cell surface proteins, including rat hepatocyte heparan sulphate proteoglycan [45], alkaline phosphatase from the surface of BC₃H1 myocytes [46], rat skeletal muscle 5'-nucleotidase [47], and a cAMP-binding ectoprotein from rat adipocytes and 3T3-L1 cells [48]. In the present study we demonstrate that the GPI-anchored MDP on the surface of 3T3-L1 cells is also released into a soluble form by insulin in a concentration- and time-dependent manner. The released form of MDP after stimulation of the cells by insulin is hydrophilic as assessed by phase separation in Triton X-114, and migrates on SDS/PAGE with the same apparent molecular mass as the form released by bacterial PI-PLC. This strongly suggests that the insulin-released MDP has been derived from the membrane-bound form through the action of a phospholipase rather than a protease, as proteolytic cleavage results in the protein migrating with a lower apparent molecular mass [49].

Indeed we provide unequivocal evidence that the form of MDP released on stimulation of 3T3-L1 adipocytes by insulin has been cleaved through the action of a phospholipase C rather than a phospholipase D, as it retains the inositol 1,2-cyclic monophosphate CRD epitope. If insulin were stimulating the

Table 2 The effect of insulin or glimepiride pretreatment of 3T3-L1 adipocytes on the subsequent release of MDP

3T3-L1 adipocytes were incubated with either insulin (100 nM) or glimepiride (50 μ M). After 15 min the medium was withdrawn and concentrated, then assayed for MDP activity (first incubation). The cells were then washed three times with KRH buffer, reincubated with PI-PLC (0.1 m-unit/ml), insulin (100 nM), glimepiride (50 μ M) or medium alone for a further 15 min before the culture medium was again withdrawn and assayed for MDP activity (second incubation). Results are means $\pm$ S.E.M. for triplicate determinations.

First incubation		Second incubation	
Treatment	MDP activity (nmol of D-Phe/min per mg)	Treatment	MDP activity (nmol of D-Phe/min per mg)
Insulin	4.1 $\pm$ 0.8	PI-PLC	5.9 $\pm$ 0.8
	3.6 $\pm$ 0.6	Insulin	0.3 $\pm$ 0.2
	3.2 $\pm$ 0.7	Glimepiride	0.5 $\pm$ 0.3
	2.8 $\pm$ 0.5	Control	0.0 $\pm$ 0.0
	2.2 $\pm$ 1.0	PI-PLC	6.5 $\pm$ 0.9
Glimepiride	2.8 $\pm$ 0.9	Insulin	1.1 $\pm$ 0.4
	2.4 $\pm$ 0.5	Glimepiride	0.5 $\pm$ 0.3
	2.1 $\pm$ 0.8	Control	0.0 $\pm$ 0.0

release of MDP through the activation of a phospholipase D, the inositol 1,2-cyclic monophosphate epitope would not be formed [17]. The cleavage of a GPI anchor by phospholipase C could lead to the generation of two second messengers, IPG and diacylglycerol. Whether the cleaved GPI anchor on the soluble form of MDP is a source of IPG awaits further studies. However, the GPI anchor on MDP in pig kidney has been shown to contain exclusively a diacylglycerol structure, predominantly distearoyl phosphatidylinositol with a minor amount of stearoyl-palmitoyl phosphatidylinositol [44], as opposed to the more common alkylacylglycerol structures present in other mammalian GPI anchors. Thus if the GPI anchor of MDP in 3T3-L1 cells also contains diacyl phosphatidylinositol, then cleavage by an insulin-stimulated phospholipase C would lead to generation of the well-characterized second messenger diacylglycerol.

Glimepiride is one of several sulphonylurea drugs used as hypoglycaemic agents in the treatment of non-insulin-dependent diabetes mellitus [50]. In addition to the action of such compounds in the pancreas, there is also evidence to indicate that they have extrapancreatic actions. Recently, it has been shown that glimepiride can stimulate the release of the GPI-anchored 5'-nucleotidase and cAMP-binding ectoprotein from the surface of 3T3-L1 cells and rat adipocytes, possibly through the action of a phospholipase C [48,51]. In the present study we show that glimepiride also stimulates the release of MDP from the surface of 3T3-L1 cells in a concentration- and time-dependent manner. Half-maximal release of MDP was observed at a concentration of 16 μ M glimepiride, which is similar to that observed for the release of 5'-nucleotidase and the cAMP-binding ectoprotein [51]. After stimulation of the 3T3-L1 cells by insulin or glimepiride, further incubation with bacterial PI-PLC resulted in a substantial release of MDP, indicating that there is still a significant fraction of MDP on the cell surface. In contrast, further incubation with either insulin or glimepiride led to the release of a greatly decreased amount of MDP. This indicates that of the total PI-PLC-releasable cell surface MDP only a subfraction is susceptible to release by insulin or glimepiride, possibly as a result of only a limited proportion of MDP being co-localized with the insulin-stimulated phospholipase C within the plasma membrane. Treatment of the cells with glimepiride after insulin, or treatment with insulin after glimepiride, led to negligible release of MDP. This would suggest that both insulin and glimepiride might be stimulating the release of the same pool of MDP, probably through activation of the same phospholipase.

The well-documented responsiveness of 3T3-L1 adipocytes to insulin and the insulin-induced release of the GPI-anchored MDP, along with the availability of the purified GPI anchor from this protein (S. P. Heywood, S. Movahedi and N. M. Hooper, unpublished work) of which the detailed structure is known [44], makes this an attractive model system in which to study the molecular mechanisms involved in the insulin-stimulated release of GPI-anchored proteins and the role of IPGs in mediating some of the actions of this hormone.

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