
Review Article

The Molecular Mechanism of the Insulin-mimetic/ sensitizing Activity of the Antidiabetic Sulfonylurea Drug Amaryl

Günter Müller

Aventis Pharma Germany, Frankfurt, Germany

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Abstract

The hypoglycemic sulfonylurea drugs cause reduction of blood glucose predominantly via stimulation of insulin release from pancreatic β cells. In addition, during long-term treatment, an insulin-independent blood glucose-decreasing mechanism is assumed to operate. This may include insulin-sensitizing and insulin-mimetic activity in muscle and adipose tissue. This review summarizes our current knowledge about the putative modes of action of the sulfonylurea compound, Amaryl, in pancreatic β cells and, in particular, peripheral target cells that form the molecular basis for its characteristic pharmacological and clinical profile. The analysis was performed in comparison with the conventional and the “golden standard” sulfonylurea, glibenclamide. I conclude: (I) The blood glucose decrease provoked by Amaryl can be explained by a combination of stimulation of insulin release from the pancreas and direct enhancement, as well as potentiation of the insulin response of glucose utilization in peripheral tissues only. (II) The underlying molecular mechanisms

seemed to rely on β cells on a sulfonylurea receptor protein, SURX, associated with the ATP-sensitive potassium channel (K_{ATP}) and different from SUR1 for glibenclamide, and in muscle and adipose cells on: (a) the increased production of diacylglycerol and activation of protein kinase C; (b) the enhanced expression of glucose transporter isoforms; and (c) the insulin receptor-independent activation of the insulin receptor substrate/phosphatidylinositol-3-kinase pathway. (III) The latter mechanism involved a nonreceptor tyrosine kinase and a number of components, such as caveolin and glycosylphosphatidylinositol structures, which are assembled in caveolae/detergent-insoluble glycolipid-enriched rafts of the target cell plasma membrane. Since hyperinsulinism and permanent K_{ATP} closure are supposed to negatively affect the pathogenesis and therapy of non-insulin-dependent diabetes mellitus, the demonstrated higher insulin-independent blood glucose-lowering activity of Amaryl may be therapeutically relevant.

Introduction

It is now evident from numerous biochemical, genetic, and clinical studies that multiple gene defects and polymorphisms, in combination with physiological consequences of the typical western life style (nutrition, fitness, obesity),

impair the functionality of the triad, β cell, muscle/adipose cell and liver cell, that regulate glucose and lipid metabolism. This results in reduced insulin secretion, reduced insulin sensitivity (insulin resistance), and increased hepatic glucose output, although their causal relationship remains a matter of intense debate. The hallmarks of increased fasting blood glucose levels, inadequate plasma insulin levels, impaired glucose tolerance, and misregulated lipid metabolism fuel a vicious cycle that, over

Address correspondence and reprint requests to:
Dr. Günter Müller, Aventis Pharma Germany, DG
Metabolic Diseases, Bldg. H825, 65926 Frankfurt am
Main, Germany. Phone: 4969-305-4271; Fax: 4969-305-
81767; E-mail: Guenter.Mueller@aventis.com

decades, leads to the development of frank diabetes with the consequence of diabetic late complications (neuropathy, retinopathy, nephropathy, micro- and macroangiopathies, and cardiovascular diseases).

Sulfonylureas have been introduced into the therapy of non-insulin-dependent diabetes mellitus (NIDDM) with great success (1). It is now generally accepted that these hypoglycemic drugs cause reduction of blood glucose predominantly via stimulation of insulin release from pancreatic β cells. In addition, during long-term treatment, an insulin-independent blood glucose-decreasing mechanism may operate. Its physiological role in diabetes therapy remains controversial and seems to be rather limited to conventional sulfonylureas (1). The predominant β -cell inherent mode of conventional sulfonylurea action may be responsible for some of the well-established, as well as hypothetical, problems with sulfonylurea therapy. The high plasma insulin levels may accelerate the pathogenesis of metabolic syndrome and NIDDM via a combination of pancreatic (exhaustion of the β cell) and peripheral mechanisms (insulin resistance, lipid disorders, obesity, increased blood pressure, and atherosclerosis). In conjunction with limited selectivity for the β cell, high plasma insulin levels may cause cardiovascular side effects. Furthermore, conventional sulfonylureas have to be administered several times per day.

In the following, I want to demonstrate that the sulfonylurea, Amaryl, despite harboring the typical sulfonylurea moiety (Fig. 1) and sharing the wide spectrum of *in vitro* (cell-free and cellular) activities attributed to conventional so-called first- (such as tolbutamide) and second-generation sulfonylureas (such as the "golden standard," glibenclamide) to a certain degree, operates at both the β -cell and cells

of peripheral tissues via distinct molecular mechanisms, compared with tolbutamide and glibenclamide. These differences may contribute to the unique pharmacological profile of Amaryl.

Interaction of Amaryl with the Pancreatic β -cell

It is now well established that increasing the closed probability of the β cell ATP-dependent potassium channel (K_{ATP}) is the major mechanism through which sulfonylureas, as well as glucose (via transport, phosphorylation, and oxidative metabolism for generation of ATP), stimulate insulin release from pancreatic β cells (2). The resulting reduction in potassium ion efflux causes depolarization of the β -cell plasma membrane, which in turn leads to opening of voltage-sensitive Ca^{2+} channels of the L-type. The increased influx of Ca^{2+} and, thus, the elevated cytosolic Ca^{2+} levels trigger the fusion of insulin-containing secretory granules with the plasma membrane (3,4). This is presumably mediated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). CaMKII is stimulated in the insulinoma cell line, MIN6, in response to secretagogues, such as glucose, the sulfonylurea tolbutamide and high K^+ , in parallel with insulin secretion (5) and phosphorylates proteins that are thought to be involved in the trafficking, docking, and fusion of insulin secretory granules (6). These include synapsin I, microtubule-associated protein-2 (MAP-2), *N*-ethylmaleimide-sensitive fusion protein (NSF), soluble NSF attachment protein (SNAP), vesicle-associated membrane protein (VAMP/synaptobrevin), α -SNAP, SNAP-25, and synaptotagmin (7) and additional unknown components that regulate the assembly/disassembly of the SNAP receptor (v/t-SNARE)/fusion protein complex. Interestingly, synapsin Ib, the major isoform in MIN6 cells was found to be associated with some isoforms of the δ -subunit of CaMKII in insulin secretory granules (8,9). This may play a critical role in the regulation of the interaction between the granules and the cytoskeleton via CaMKII-dependent phosphorylation of synapsin I.

The β -cell K_{ATP} consists of the regulatory sulfonylurea receptor subunits (SUR), such as SUR1, and the physically associated pore-forming potassium ion inwardly rectifying subunit, KIR6.2, in a multimeric assembly

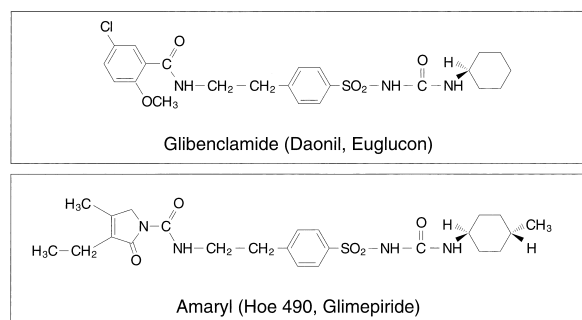


Fig. 1. Structures of Amaryl and glibenclamide.

with the SUR and KIR6.2 subunits in 1:1-stoichiometry and four identical SUR/KIR6.2 complexes per functional channel unit [size of the channel holocomplex [SUR/KIR6.2]₄ about 1000 kDa (10–12)]. SUR1, which is predominantly expressed in glucose-responsive neurons of the hypothalamus and pancreatic β cells, is an ATP-binding cassette (ABC) protein or transport ATPase, which closely resembles members of the multidrug resistance-associated protein family. It harbors 17 predicted transmembrane domains (TMD) and two nucleotide-binding folds (NBF), each constituted by a Walker motif A and B, which bind specifically (Mg^{2+})ADP/ATP (Fig. 2) (13–15). The KIR6.2 subunits have two TMDs that somehow contribute to the K^+ conductivity and selectivity (16). The glibenclamide-binding site of SUR1 is proposed to consist of a benzamido (meglitinide)-binding site on TMDs 1–5 and the sulfonylurea (tolbutamide)-binding site on TMDs 12–17, based on photolabeling with [¹²⁵I] iodoglibenclamide and chimeric receptors (17,18). Interaction with both the meglitinide- and tolbutamide-binding sites could account for the several thousand-fold increase in affinity of glibenclamide (equipped with both a benzamido and sulfonylurea moiety) vs. tolbutamide (equipped with a sulfonylurea moiety, only). SUR2A/B are splice variants of a single

SUR2 gene that exclusively differ in 42 amino acids at the carboxy-terminus, with SUR2A expressed mainly in cardiac/skeletal muscle cells and SUR2B in vascular/nonvascular smooth muscle cells (19). K^+ -channel openers (KCO) bind to SURs at the tolbutamide-binding site, encompassing those (flanking) TMDs 12–17 that surround the central core region of the tolbutamide-binding site (20). SURs define the sensitivity of the K_{ATP} holocomplex for its sensitivity toward both sulfonylureas and KCOs with SUR1 mediating high sensitivity for sulfonylureas/low sensitivity toward KCOs and, vice versa, SUR2A/2B mediating low sensitivity for sulfonylureas/high sensitivity for KCOs. Chimeric SURs can be engineered by recombinant DNA technology (harboring TMDs 12–17 from both SUR1 and SUR2A/B), with high sensitivity for both sulfonylureas and KCOs, arguing for a modular structural and functional organisation of SURs (20).

The cooperative interactions between NBFs 1 and 2 of SUR1 in the open state of the K_{ATP} are disrupted by the conformational change induced by glibenclamide-binding that encompasses TMD 12–17 and, especially, TMD1–5 (Fig. 3) (18). This conformational change repositions TMD 2 of KIR6.2 (M2), which is in close contact to TMD 1–5 of SUR1, to a closed state via a mechanism that requires the intact

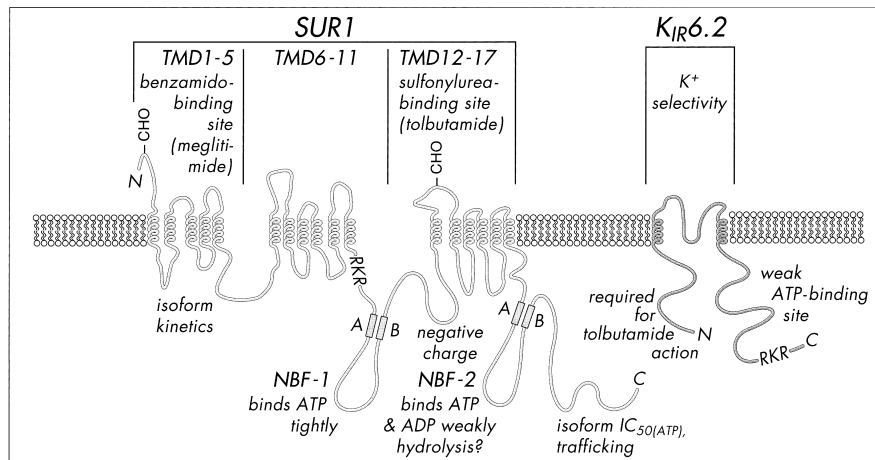


Fig. 2. Mapping of known properties of K_{ATP} to segments and structural features of SUR and KIR6.2. [See text for details (18)]. The -RKR- motif has been demonstrated to function as quality control (protein trafficking) signal in keeping incompletely assembled K_{ATP} (i.e. incorrect stoichiometry) from reaching the cell surface. This motif is found in the carboxy-terminus of subunit KIR6.2 and in sulfonylurea receptor protein for glibenclamide (SUR1) upstream of

nucleotide-binding folds (NBF1), mutation of which leads to cell surface expression in the absence of the partner subunit (139). It may function as endoplasmic reticulum retention signal that is masked during assembly of the channel complex, thus, allowing only correctly assembled and functional (i.e. with regard to regulated conductivity) K_{ATP} to reach the plasma membrane. CHO, putative glycosylation sites. TMD, transmembrane domains.

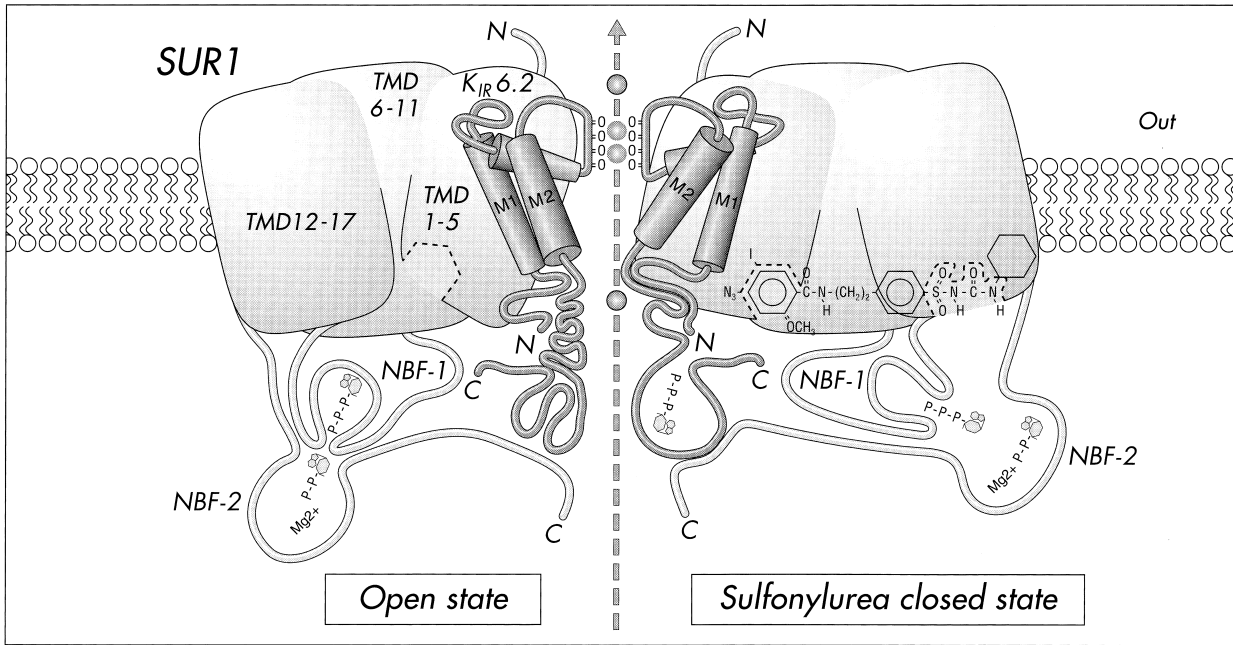


Fig. 3. Hypothetical model for the open and glibenclamide-induced closed states of a K_{ATP} . [See text for details (18)]. In the glibenclamide-bound state (right section), interaction of the inhibitory ATP with subunit $K_{IR6.2}$ is indicated; whereas, ATP and Mg^{2+} ADP (the interaction of

which with the nucleotide-binding folds (NBFs) of sulfonylurea receptor protein for glibenclamide (SUR1) in the open state is shown on the left section) are leaving NBF-1 and NBF-2, respectively, of SUR1. TMD, transmembrane domains.

amino-terminus of $K_{IR6.2}$. Thus, sulfonylurea-binding to SUR1 apparently induces a subtle relaxation of the intimate association between SUR1 and $K_{IR6.2}$, which is required for keeping $K_{IR6.2}$ in a (partly) open state. Compatible with this simplified view are recent findings that certain mutations in both SUR1 and $K_{IR6.2}$ cause recessive forms of familial hyperinsulinism or persistent hyperinsulinemic hypoglycemia of infancy due to permanent closure of K_{ATP} , presumably in the course of weakening the interaction between SUR1 and $K_{IR6.2}$ to a certain degree (21–23).

Binding of ATP to the carboxy-terminal domain of $K_{IR6.2}$ facilitates and/or stabilizes the glibenclamide-induced relative dissociation of SUR1 and $K_{IR6.2}$, possibly mediated by its proximity to the amino-terminus of $K_{IR6.2}$, which is accomplished during repositioning of M2 (see above). Thereby, ATP-binding favours the closed state of the K_{ATP} . This may explain, in part, the (synergistic) stimulation of glucose-induced (i.e. ATP-mediated) insulin release by sulfonylureas. In contrast, interaction of ATP with NBF-1 and Mg^{2+} ADP with NBF-2 of SUR1 is relieved upon glibenclamide-binding (see Fig. 3), compatible with Mg^{2+} ADP-binding to

NBF1/2 of SUR1 triggering opening of $K_{IR6.2}$. Site-directed mutations in a single NBF are sufficient to block the stimulatory effect of Mg^{2+} ADP. These mutations also prevent K_{ATP} activation by metabolic inhibition in intact cells (24,25). This suggests an important role of SUR1 and its NBFs, in particular, for metabolic regulation of the K_{ATP} in response to low energy charge of the cell. Furthermore, mutations associated with persistent hyperinsulinemic hypoglycemia of infancy have been identified in SUR1 that impair the ability of Mg^{2+} ADP to enhance K_{ATP} channel activity, but do not change the inhibitory effect of ATP (25,26). These mutations abolish the ability of $K_{IR6.2}$ to respond to changes in the oxidative glucose metabolism and cause it to be permanently closed in the intact β cell. These findings suggest that the physiological role of SUR1 seems to rely on the mediation of metabolic regulation of channel activity in response to nutritional deprivation. The binding of sulfonylureas to SUR1 may be simply providential.

Amaryl and glibenclamide bind to SURs of the β cell with high specificity and affinity (27,28). In kinetic experiments, the association and dissociation rate constants, K_{on} and K_{off}

values, of Amaryl are 2.5- to 3-times and 8- to 9-times, respectively, higher than those of glibenclamide, using both membranes from solid New England Deaconess Hospital (NEDH) rat β -cell tumor or cultured rat insulinoma m5F (RINm5F) cells and intact cultured β -cells. The calculated 3- to 4-fold higher K_{off}/K_{on} -ratios of Amaryl, compared with glibenclamide, were identical to the dissociation constants (K_d -values) as determined by Scatchard plot analysis under steady state conditions with all three sources of the receptors. The 3- to 4-fold lower binding affinity of Amaryl, compared with that of glibenclamide, was also reflected in homologous and heterologous competitions studies (27,28). Three- to 4-fold higher concentrations of unlabeled Amaryl, compared with glibenclamide, were required for half-maximal displacement of both radiolabeled Amaryl and glibenclamide from specific binding to membranes of cultured RINm5f cells. The lower binding affinity of Amaryl, compared with glibenclamide, correlates well to their depolarization activity (Fig. 4). In the presence of KCO (100 μ M diazoxide), the inhibitory concentrations (IC_{50}) for depolarization of cultured RINm5F cells in the whole-cell patch-clamp configuration were 27.3 nM for Amaryl and 7.4 nM for glibenclamide.

For elucidation of the molecular basis for the distinct binding and depolarisation characteristics of Amaryl, our lab performed direct photoaffinity labeling of β -cell membranes from diverse sources with unmodified radiolabeled Amaryl or glibenclamide (29,30). As

reported previously (31), [3 H]glibenclamide was crosslinked to a 140-kDa polypeptide, which corresponded to SUR1, and to an unidentified 33-kDa polypeptide. In contrast, a 65-kDa polypeptide, SURX, exclusively was radiolabeled by [3 H]Amaryl using both isolated β -cell tumor membranes and intact cultured β cells for the photocrosslinking. Apparently, Amaryl and glibenclamide interacted with different sulfonylurea receptor proteins, SURs, of the β -cell plasma membrane. Surprisingly, an excess of unlabeled glibenclamide or tolbutamide included during the irradiation reaction completely inhibited photolabeling of SURX (65 kDa) by [3 H]Amaryl and, vice versa, unlabeled Amaryl reduced photolabeling of SUR1 (140 kDa) by [3 H]glibenclamide in a concentration-dependent fashion with similar efficiency as glibenclamide (29). Thus, SUR1 and SURX seem to interact with one another in a cooperative fashion.

Using similar techniques, it has been demonstrated in earlier studies that glibenclamide, in addition to specific binding to the 140-kDa protein, specifically interacts with about 40- and 65-kDa polypeptides (32,33). Thus, it cannot be excluded that glibenclamide also binds to SURX, albeit with significantly lower affinity than Amaryl. Alternatively, different ligands, such as Amaryl and glibenclamide, may recognize SURX in different conformational states, which would affect the binding affinity and selectivity of either Amaryl or glibenclamide. This would explain the distinct patterns of photoaffinity labeling and equilibrium/kinetic binding. Nevertheless, on

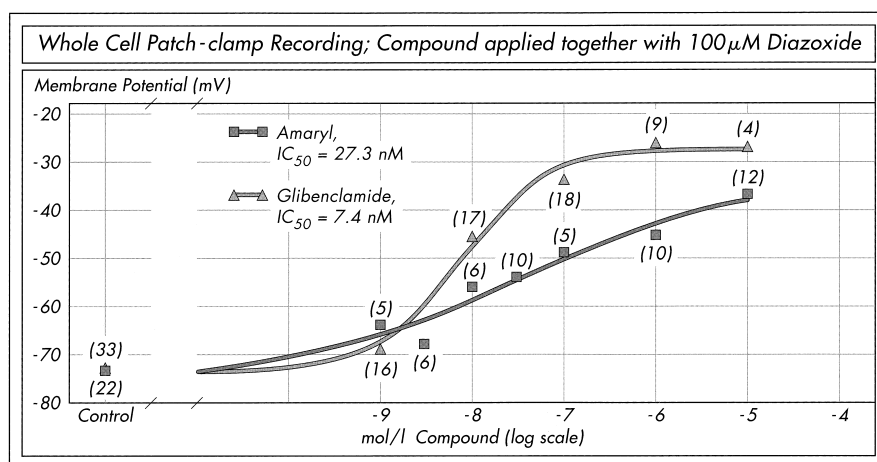


Fig. 4. Effect of Amaryl and glibenclamide on the diazoxide-activated membrane potential of cultured rat insulinoma m5F (RINm5F) cells. The numbers of measurements at each concentration are indicated. IC_{50} , inhibitory concentrations.

basis of the (I) unambiguous photolabeling of SUR1 by glibenclamide, but not Amaryl under the same experimental conditions and (II) considerably different (albeit overlapping) binding characteristics of the two drugs, different SURs, i.e. SUR1 and SURX, may be proposed as the major specific binding proteins for glibenclamide and Amaryl, respectively, in the β cell.

These data and considerations together suggest the following structural model for the β -cell K_{ATP} . Conventional sulfonylureas, such as glibenclamide, and the novel sulfonylurea, Amaryl, bind to distinct receptor subunits, the well-characterized SUR1, and SURX (identified so far by photoaffinity labeling only), respectively. SUR1 and SURX interact with each other in a cooperative fashion and control the open/closed probability of the associated pore-forming subunits, KIR6.2, SUR1 independent of SURX, and SURX in close coupling to SUR1 only. In the putative functional 12-meric channel complex consisting of four identical SUR1/SURX/KIR6.2 trimers, (SUR1/SURX/KIR6.2)₄, and embedded in the intact β -cell membrane, Amaryl and glibenclamide manage to bind to and photolabel SURX and SUR1, respectively; whereas, upon membrane solubilisation by detergents and concomitant complex dissociation, both Amaryl and glibenclamide interact with SURX, exclusively (29).

The Insulin-independent Blood Glucose-Decreasing Activity of Amaryl in Animals

Despite the lower β -cell activity of Amaryl vs. glibenclamide in terms of binding affinity and duration of binding, as well as depolarization, clinical studies reveal a higher blood glucose-lowering activity of Amaryl in man, because the doses required for equivalent blood glucose decreases are 50–70% of those of glibenclamide (Fig. 5) (34–38). This is in striking contrast to the conventional view that the blood glucose-decreasing activity of sulfonylureas is based solely on the stimulation of insulin release from the pancreatic β cell. Since the introduction of sulfonylureas into the therapy of NIDDM, a controversial debate has emerged as to whether these oral hypoglycemic drugs, in addition to their capacity for inducing insulin release, have an insulin-independent blood glucose-decreasing activity, such as an activity that does not require additional insulin release.

	Amaryl	Glibenclamide
● Binding affinity	1	3 - 4
● Duration	1	2.5 - 3.5
● Depolarization Potency	1	3 - 3.5
nevertheless		
● Dose for equivalent blood glucose decrease in humans	1/3 - 1/2	1

Fig. 5. Comparison of Amaryl and glibenclamide for efficiency at the β cell K_{ATP} and hypoglycemic activity. The K_d (6.8 nM) and association rate constant, K_{on} ($0.5 \text{ min}^{-1}/\text{nM}^{-1}$), values for binding and the inhibitory concentrations (IC_{50}) for depolarization (27.3 nM) determined for membranes of cultured rat insulinoma m5F (RINm5F) cells or intact cells, respectively, were set at 1 for Amaryl, the dose required for equivalent blood glucose decrease in humans was set at 1 for glibenclamide.

The observation that after chronic treatment of NIDDM patients with sulfonylureas, their plasma insulin levels return to near pretreatment levels without concomitant loss of hypoglycemic control has been explained by insulin-independent activities of these drugs exerted at both pancreatic and extrapancreatic tissues (1).

To re-evaluate the possibility of an insulin-independent blood glucose-decreasing activity of sulfonylureas, in general, and of Amaryl, in particular, we reason that the ratio of the sulfonylurea-induced mean plasma insulin release and mean blood glucose decrease measured over the total study period should be identical for structurally different sulfonylureas, provided the time response curves for the insulin release and blood glucose decrease exhibit similar shapes and the blood glucose decrease is based solely on the stimulation of insulin release. Different ratios for structurally different sulfonylureas would argue in favour of the operation of an additional insulin-independent blood glucose-decreasing activity. To test this hypothesis, in four different experiments, Amaryl and the sulfonylureas, glibenclamide, gliclazide and glipizide, were tested in normal fasted beagle dogs at submaximal single oral or i.v. doses, which lower blood glucose nadir levels to about the same degree for their blood glucose-lowering and plasma insulin-releasing activity (39,40). For instance, after single oral administration of different doses of these sulfonylureas, Amaryl (90 $\mu\text{g}/\text{kg}$)

had the highest total blood glucose-lowering activity and the lowest insulin-releasing activity during the 36 hr post-treatment period; whereas, glipizide (180 $\mu\text{g}/\text{kg}$) had the lowest total blood glucose-lowering activity, and highest insulin-releasing activity, and glibenclamide (90 $\mu\text{g}/\text{kg}$) and gliclazide (1.8 mg/kg) ranked between the two extremes. For calculation of the plasma insulin/blood glucose ratio by means of the trapezoidal rule for each animal, the mean plasma insulin increase and mean percentage blood glucose decrease over the whole study period in fasted male beagle dogs after a single oral or i.v. dose of the different sulfonylureas were determined. Single-dose studies over periods of 0–8 hr to up to 0–51 hr were preferred to exclude possible indirect effects of prolonged sulfonylurea treatment secondary to the decreased basal blood glucose and plasma insulin levels via improvement of the metabolic overall situation and/or insulin sensitivity.

In these studies, the shapes of the blood glucose and plasma insulin curves and the blood glucose nadirs were approximately identical. However, for a given experiment, the ratios differed for different sulfonylureas (e.g. Amaryl, 0.03; gliclazide, 0.07; glipizide, 0.11; and glibenclamide, 0.16). Within each experiment there was an identical ranking of the ratios of the mean plasma insulin release/mean blood glucose decrease among the various sulfonylureas. Amaryl exhibited the lowest ratio in each experiment. This was due primarily to a statistically significant lower plasma insulin release (with respect to both maximal and mean plasma insulin levels) in response to Amaryl, compared with the other drugs (e.g. mean plasma insulin levels: Amaryl, 0.6 $\mu\text{U}/\text{ml}$; gliclazide, 1.3; glipizide, 1.6; and glibenclamide, 3.3). The differences and the ranking in the plasma insulin/blood glucose ratios suggest that sulfonylureas have insulin-independent blood glucose-decreasing activity and that this is more pronounced for Amaryl than for conventional sulfonylureas.

This interpretation could be strengthened by a number of additional animal experiments, the results of which are not compatible with insulin release as the only mechanism for sulfonylurea-induced blood glucose decrease (39,40). (I) Administration of higher single oral and i.v. doses (60 $\mu\text{g}/\text{kg}$) to normal fasted rabbits provoked approximately identical time response curves for the blood glucose decrease

up to 8 hr for Amaryl and glibenclamide. However, during the following 40 hr, the Amaryl animals showed significantly lower blood glucose levels; thus, apparently, Amaryl had the longer-lasting blood glucose-decreasing activity. Remarkably, at these later time points after both i.v. or oral administration (20–48 hr), there was still a pronounced blood glucose decrease induced by Amaryl and the plasma insulin levels of the Amaryl animals already had decreased to near basal levels. (II) In normal fasted beagle dogs treated with a single oral dose of 90 $\mu\text{g}/\text{kg}$ of Amaryl or glibenclamide and, subsequently (30 min later), repeatedly loaded orally with glucose (2 g/kg) in 5 hr intervals for up to 50 hr, the mean blood glucose concentrations over the whole study period in the Amaryl and glibenclamide animals were significantly lower than those in the controls. However, the mean plasma insulin values also were lower in both drug groups, with the Amaryl animals exhibiting significantly lower mean plasma insulin levels than the glibenclamide animals. (III) In manifestly diabetic KK-A^y mice, a typical type II diabetes animal model, Amaryl (0.5 mg/kg/day) reduced the blood glucose and plasma insulin levels by 50% and the glycated hemoglobin (HBA_{1C}) by 33% after 8 weeks of once-daily treatment. Glibenclamide (1.25 mg/kg/day) and gliclazide (20 mg/kg/day), at equipotent blood glucose-decreasing doses in rabbits, had no effect on blood glucose, plasma insulin, and HBA_{1C} under these experimental conditions.

The described pharmacological data can only be explained by the assumption that an insulin-independent blood glucose-decreasing activity operates at the level of pancreatic and/or extrapancreatic tissues during sulfonylurea action, in general, and Amaryl action, in particular. Possible mechanisms include inhibition of glucagon release from pancreatic α cells, interference with insulin removal by the liver, facilitation of transendothelial transport of insulin, increase of insulin sensitivity in peripheral tissues, and direct stimulation of glucose utilization in muscle and fat tissues. There is some experimental evidence that sulfonylureas may affect all these processes to some extent, e.g. attenuation of glucagon secretion by Amaryl (41). However, a number of in vitro studies with isolated and cultured insulin-sensitive fat and muscle cells that used well-defined incubation conditions in the presence of Amaryl and/or glibenclamide have

been performed during the last decade (42–50). Most data argue for an improvement of insulin sensitivity and an increase of glucose utilization in peripheral tissues as the major potential mechanisms for blood glucose decrease by sulfonylureas, in addition to insulin release (42–50). In vivo, these mechanisms will lead to increased glucose flux from the blood into muscle and fat tissues, even in the absence of (additional) insulin. Therefore, the following part of the review is dedicated to the underlying molecular basis. As a prerequisite for the in vivo relevance of a mechanism identified in vitro, the ranking of the potency ratios [determined as the ratio of equi-effective concentrations of the two drugs derived from the respective graded (by parallel line assay) concentration-response relations] between different sulfonylureas calculated from the in vitro data should correspond to that of their insulin release/blood glucose decrease ratios in vivo.

Direct Stimulation of Glucose Utilization by Amaryl in Fat and Muscle Cells In Vitro

The major pathway for rapid glucose utilization is the non-oxidative glucose metabolism with the two branches of glycogen and lipid synthesis. Therefore, the effects of Amaryl and glibenclamide on glycogenesis and lipogenesis in the isolated rat diaphragm and cultured mouse 3T3 adipocytes, respectively, were studied (42). Both drugs concentration-dependently stimulated glycogenesis up to 2.5-fold and lipogenesis to up to 4.2-fold (at 20 μM) above basal after 4 hr and 20 hr incubation, respectively. The maximal responses (synthesis rates) did not differ between Amaryl and glibenclamide. But, there was a statistically significant leftward-shift of the concentration-response curves for Amaryl, compared with those for glibenclamide. This resulted in about 50% reductions of the corresponding effective concentrations (EC_{50}), which were in the 1 to 5 μM range. At low glucose concentrations, the rate of lipogenesis and glycogenesis in vivo is controlled by the glucose transport step. Both drugs stimulated concentration-dependently the transport of the nonmetabolizable glucose analog, 2-deoxyglucose, into 3T3 adipocytes up to 6.8-fold (at 20 μM) and in rat diaphragms up to 1.8-fold (at 20 μM) (42). Again, Amaryl

and glibenclamide showed the same maximal responses, but with a potency ratio of 2.4 and 1.8, respectively, in favour of Amaryl.

NIDDM is related causally to resistance of the glucose transport system for stimulation by insulin. This insulin-resistant state can be mimicked in vitro by preincubation of isolated rat adipocytes with high concentrations of insulin, glutamine, and glucose in primary culture. This mimicks the diabetic milieu to which tissues are exposed during the pathogenesis of NIDDM. When these cells were assayed subsequently for insulin sensitivity in low glucose-containing medium, the maximal insulin-stimulated glucose transport (at 10 nM) was reduced by about 60% and the EC_{50} for insulin was increased 3- to 3.5-fold, compared with control cells (51). In contrast, the maximal response (at 10 μM) and the EC_{50} for Amaryl hardly were affected by the insulin-resistant state, resulting in concentration-response curves that were virtually identical for normal and insulin-resistant adipocytes. Apparently, Amaryl activates the glucose transport system in (insulin-resistant) isolated rat adipocytes by interference at a site downstream of the putative insulin resistance defect. Insulin stimulates glucose transport in fat and muscle cells by increasing the cell surface expression of the glucose transporter isoforms, GLUT4, and to a minor degree, GLUT1 (52). Upon insulin binding, the autophosphorylated insulin receptor activates the downstream signaling cascade, which via a complex network of phosphorylations of signaling molecules and metabolic enzymes, as well as interactions between them, induces lipid and glycogen synthesis and triggers the fusion of intracellular GLUT-containing vesicles with the plasma membrane, the so-called GLUT4/1 translocation (53,54). After incubation of normal isolated rat adipocytes for 20 min with 10 nM insulin, the amount of GLUT4 at the plasma membrane was raised 6.5- to 7.5-fold, accompanied by a corresponding decrease of GLUT4 in intracellular vesicles (51). In insulin-resistant adipocytes, the insulin-stimulated GLUT4 translocation was reduced 1.8- to 2.2-fold. Amaryl, however, stimulated GLUT4 translocation up to 2.5- to 3.0-fold in both normal and insulin-resistant adipocytes. Similarly, the (less pronounced) GLUT1 translocation was reduced considerably in insulin-resistant adipocytes, compared with normal adipocytes in response to insulin, but not Amaryl (51). Thus, GLUT translocation is

part of the molecular basis for the efficient glucose transport stimulation by Amaryl in both normal and insulin-resistant cells.

Regulation of Key Metabolic Proteins/Enzymes by Amaryl in Adipose Cells

Phosphorylation of GLUT4 is one potential mechanism for regulation of the distribution of GLUT4 between the plasma membrane and the intracellular GLUT4 vesicles. This is compatible with our finding that, after induction of insulin resistance in isolated rat adipocytes, the phosphorylation state of GLUT4 was significantly increased, compared with that of normal cells (51). Treatment of insulin-resistant adipocytes with insulin (10 nM for 20 min) reduced the amount of phosphorylated GLUT4 by 30–40%, and treatment with Amaryl (10 μ M), by 70–80%. Since GLUT4 is already in the phosphorylated state after full desensitization of the adipocytes, Amaryl obviously activates a (presumably serine-/threonine-specific) protein phosphatase dephosphorylating GLUT4, rather than inhibits a GLUT4-specific protein kinase. The dephosphorylation may reduce the internalization rate of GLUT4. The impaired internalization, in conjunction with the constitutive externalization of GLUT4 even in the absence of insulin, as was demonstrated for adipocytes (53), ultimately will lead to a net flux of GLUT4 molecules from the intracellular GLUT4 vesicles to the plasma membrane. The Amaryl-induced GLUT4 dephosphorylation and, in consequence, translocation may be particularly prominent in insulin-resistant adipocytes, due to their high content of phosphorylated GLUT4 (51). In addition to this dephosphorylation mechanism as the molecular basis for Amaryl-induced GLUT translocation, studies with isolated rat cardiomyocytes demonstrated the induction of GLUT1 and GLUT4 expression by Amaryl (55). The total amount of GLUT1 and 4 protein increased 1.7- and 1.5-fold, respectively, in response to 20 hr incubation with 10 μ M Amaryl. This correlates well with the approximate 2-fold increase in basal, as well as insulin-stimulated 2-deoxyglucose transport. Apparently, in cardiomyocytes, the Amaryl-induced GLUT1/4 gene/protein expression is the molecular basis for direct (insulin-mimetic) stimulation, as well as potentiation of the insulin stimulation of GLUT 1/4 translocation and glucose transport by this drug (56).

At high glucose concentrations, such as in the diabetic state, glycogen synthase (GS) and glycerol-3-phosphate acyltransferase (GPAT) are rate-limiting for glycogenesis and lipogenesis, respectively, rather than glucose transport. Both enzymes are stimulated by Amaryl and glibenclamide in a concentration-dependent fashion (42). GS activity increased up to 5.5-fold (at 20 μ M) after 4 hr incubation in the isolated rat diaphragm; GPAT activity up to 1.9-fold (at 20 μ M) after 20 hr incubation in 3T3 adipocytes. The potency ratios of 3.5 and 1.9, respectively, are in favour of Amaryl. Both enzymes are known to be inhibited by phosphorylation by cAMP-dependent protein kinase A (PKA). Amaryl stimulated the particulate (microsomal) cAMP-specific cGMP-inhibitable phosphodiesterase (presumably PDE3B) in a concentration-dependent fashion up to 2.3-fold (at 10 μ M) in isolated rat adipocytes, thus lowering cytosolic cAMP levels. Furthermore, Amaryl reduced cytosolic PKA activity by up to 30% (at 20 μ M), even in the presence of excess of cAMP, and thus in a cAMP-independent manner (42). For both effects, which ultimately will lead to reduced cAMP-dependent phosphorylation of GS and GPAT and, in consequence to their activation, the potency ratios were in slight favour of Amaryl, compared with glibenclamide.

In addition to the inhibition of cAMP-dependent phosphorylation, evidence was found for the activation of protein phosphatases partly mediating the stimulatory effects of sulfonylureas on glycogenesis and lipogenesis (Fig. 6). Activation of GS and GPAT by Amaryl in isolated rat diaphragms and adipocytes, respectively, was blocked completely in the presence of the serine-/threonine-specific protein phosphatase inhibitor, okadaic acid (100 nM). The proposed Amaryl-induced dephosphorylation of key metabolic enzymes of glucose and lipid metabolism was demonstrated directly for GS in isolated rat adipocytes. Amaryl caused a significant concentration-dependent reduction in the phosphorylation of GS, which was more potent compared with that provoked by glibenclamide. Insulin also lowered the phosphorylation state of GS and was most effective. In addition to PKA, glycogen synthase kinase 3 (GSK-3) phosphorylates and, thereby, inhibits GS. Compatible with the involvement of GSK-3, we found that GSK-3 activity (measured as autophosphorylation) was down-regulated in

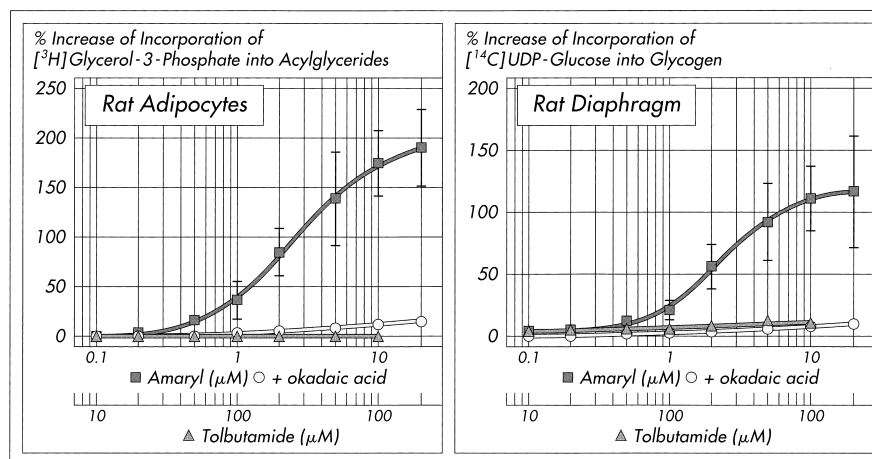


Fig. 6. Effect of okadaic acid on the activation of key metabolic enzymes by Amaryl in rat adipocytes and diaphragms. Isolated rat adipocytes or diaphragms were treated with 100 nM okadaic acid for 5 min at 30°C prior to incubation with increasing concentrations of Amaryl or tolbutamide for 30 min. For determination of glycerol-3-phosphate acyltransferase (GPAT) activity, total microsomes isolated from the cell homogenates were incubated with [³H]glycerol-3-phosphate for 3 min at 25°C. For determination of glycogen synthase (GS) activity, a

post-mitochondrial supernatant prepared from the cell homogenates was incubated with [¹⁴C]UDP-glucose for 10 min at 30°C. The activities were calculated from the radiolabeled acylglycerides in the butanol phase for GPAT and radiolabeled glycogen in the ethanol precipitate for GS as described previously (42,136). The specific activity (difference between presence and absence of enzyme source) in the absence of drug was set at 0% in each case. Each point represents the means \pm standard deviation (SD) of (at least) three independent experiments.

response to both the sulfonylureas, Amaryl and glibenclamide, and insulin in isolated rat adipocytes. Tolbutamide failed to significantly diminish both GS phosphorylation and GSK-3 autophosphorylation. GSK-3 is a key regulatory enzyme of glycogen metabolism and, in addition, a key signaling component of the insulin signal transduction cascade located downstream, presumably at the site of signal divergence to the metabolic and mitogenic pathways (57).

Insulin-mimetic Signaling of Amaryl in Adipose Cells

The demonstrated stimulatory effects of Amaryl on nonoxidative glucose metabolism (i.e. glucose transport, GLUT4 translocation, lipid and glycogen synthesis, GPAT and GS activity) and dephosphorylation, resemble the pleiotropic metabolic insulin actions that are mediated by the insulin signal transduction cascade (57). The insulin signal generated by insulin receptor kinase-mediated tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins (58) is thought to diverge to the various termi-

nal metabolic effector systems, such as GLUT4, GPAT, and GS, at sites downstream of phosphatidylinositol-3-kinase (PI-3K), protein kinase B (PKB), and GSK-3 (59). Therefore, we studied a possible interference of Amaryl with insulin signaling components located upstream of GSK-3.

GSK-3 is phosphorylated and, thereby, inhibited by PKB. In isolated rat adipocytes, Amaryl and, to a minor degree, glibenclamide, but not tolbutamide, stimulated in a concentration-dependent fashion PKB activity up to 3.6- and 2.3-fold, respectively (at 20 μ M; G. Müller, S. Welte, N. Hanekop, manuscript submitted). PKB is phosphorylated (by phosphoinositide-dependent kinases 1 and 2) and, thereby, activated in response to active PI-3K (60,61). PI-3K activity was increased in a concentration-dependent fashion in response to glibenclamide and, more potently, Amaryl up to 3.1- and 4.4-fold (at 20 μ M), respectively. During insulin action, PI-3K becomes activated by binding to tyrosine-phosphorylated IRS proteins (62,63). Interestingly, in rat adipocytes, Amaryl caused concentration-dependent tyrosine phosphorylation of IRS-1 and 2, and association of PI-3K with IRS-1 and 2. Tyrosine phosphorylation of

IRS-1 and 2 and their association with p85 of PI-3K were more pronounced with Amaryl (EC_{50} about $3 \mu\text{M}$), compared with glibenclamide; whereas, tolbutamide was ineffective (G. Müller, S. Welte, N. Hanekop, manuscript submitted). The principle IRS involved in metabolic regulation are IRS-1 and IRS-2, and there is abundant biochemical evidence to support this view (62,64). Genetic evidence from animals devoid of IRS-1 (65,66) and IRS-2 (67) further suggests that these molecules are intimately involved with the signaling to metabolic pathways regulated by insulin. Two additional members of the IRS family, IRS-3 and IRS-4, have been described, but they are unlikely to be involved in insulin-dependent metabolic regulation, because the latter is not expressed in the appropriate tissues (68) and a knockout of the former has no obvious metabolic phenotype (69). IRS-1 and 2 contain numerous tyrosine residues that, when phosphorylated, act as docking sites for a certain set of Src homology 2 domain-containing proteins. In particular, it is the activation of PI-3K in association with tyrosine-phosphorylated IRS-1 and 2 that propagates the insulin signaling cascade required for most or all of insulin's pleiotropic actions, including metabolic regulation.

Insulin provokes tyrosine phosphorylation of IRS proteins by activation of the insulin receptor tyrosine kinase β -subunit (57,64). In contrast, Amaryl has no direct effect on the insulin receptor. Takada and coworkers (70) did not find any significant effect of Amaryl on the number and binding affinity of skeletal muscle insulin receptor in genetically diabetic KK- A^y mice as well as on autophosphorylation of the human insulin receptor heterologously expressed in rat fibroblasts. This raises the question of how Amaryl manages to induce tyrosine phosphorylation of the IRS proteins in adipose and muscle cells. Interestingly, we identified another protein, caveolin, which became tyrosine-phosphorylated in isolated rat adipocytes in response to concentrations of Amaryl that were effective for stimulating glucose transport (40). Tyrosine phosphorylation of this 29-kDa plasma membrane protein was increased up to 4.2-fold above basal (at $5 \mu\text{M}$). Remarkably, treatment of intact rat adipocytes with insulin or a bacterial phosphatidylinositol (PI)-specific phospholipase C (PLC) also caused tyrosine phosphorylation of caveolin up to 9.2- (at 2 nM) and 2.1-fold (at $10 \mu\text{U/ml}$), respectively (40).

The PI-PLC cleaves glycosyl-phosphatidylinositol (GPI) lipids. They exist either in free form or as membrane anchors covalently bound to GPI-anchored proteins (GPI proteins). GPI proteins are cell surface ectoproteins of eucaryotic cells and embedded in the outer leaflet of the plasma membrane by the covalently linked GPI moiety (71), such as the cAMP-binding ectoprotein, Gce1, of rat and mouse adipocytes (72,73). Many cell types, including adipose and muscle cells, harbor an endogenous GPI-specific PLC (GPI-PLC) that, upon activation, causes the release of the protein moiety of GPI proteins from the cell surface into the cellular environment due to lipolytic cleavage of its GPI anchor (74,75). In isolated and cultured adipocytes, certain GPI proteins, such as lipoprotein lipase (72), are released from the cell surface into the incubation medium by the action of an endogenous GPI-PLC that is activated by both insulin (74–77) and Amaryl (73,76,77). In normal rat adipocytes, Amaryl activated the GPI-PLC concentration-dependently by up to 30% of the maximal insulin effect ($3 \mu\text{M}$) after 4 hr incubation. In insulin-resistant adipocytes, stimulation of the enzyme by insulin was reduced drastically (by 55–65%, compared with normal cells); whereas, activation by Amaryl was not diminished significantly (76). In contrast to the insulin effect, which required 15 min only, for full stimulation up to 14.5-fold above basal (at 10 nM), activation of the GPI-PLC by Amaryl in vitro was dependent strictly on the incubation time with a maximum of up to 9-fold (at $5 \mu\text{M}$) after 4 hr as demonstrated in rat adipocytes for the cleavage of the GPI anchor of Gce1 (conversion of the uncleaved amphiphilic to the lipolytically cleaved hydrophilic version; Fig. 7). Tolbutamide, at 100-fold higher concentrations, was completely ineffective even after 4 hr incubation.

Interaction of Amaryl with Caveolae/DIGs

Caveolin is tyrosine-phosphorylated in response to Amaryl. GPI-PLC is activated in response to Amaryl. GPI lipids/proteins are cleaved lipolytically in response to Amaryl. All of these elements are not equally distributed over the cell surface. Rather, they appear to be clustered together with another marker protein, flotillin, and other glycolipids, glycosphingolipids, cholesterol, and lipid-modified signaling proteins (such as heterotrimeric and small G-proteins,

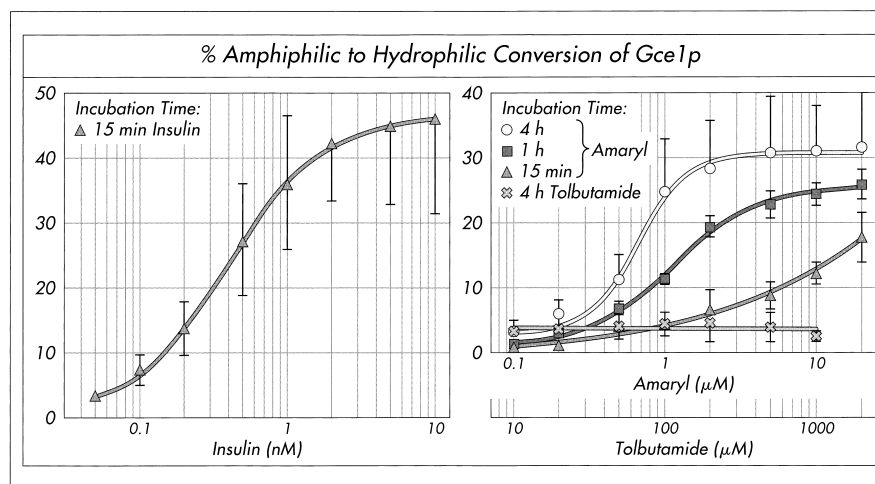


Fig. 7. Time-dependent activation of the glycosyl-phosphatidylinositol-phospholipase C (GPI-PLC) by Amaryl and insulin in rat adipocytes. Isolated rat adipocytes were photoaffinity-labeled with N_3 - $[^{32}P]cAMP$ (72,73) and then incubated with increasing concentrations of different sulfonylureas or human insulin for the periods indicated at 30°C. Plasma membranes were prepared by differential centrifugation and then

subjected to partitioning between an aqueous and a TX-114 phase. Both phases were measured for radioactivity by liquid scintillation counting. The radioactivity contained in the TX-114 phase due to the presence of the uncleaved amphiphilic version of the GPI protein, Gce1, at time point 0 in the absence of agent was set at 100%. Each point represents the mean \pm standard deviation (SD) of (at least) four independent experiments.

acylated non-receptor tyrosine kinases, protein kinase C isoform α , and certain receptors and transporters) in special areas of the plasma membrane, the so-called caveolae (78). Caveolae are small bulb-/flask-shaped invaginations of the plasma membrane present in most cell types, but highly abundant in terminally differentiated cells such, as adipocytes (20% of the total plasma membrane surface area), myocytes, epithelial, and endothelial cells. These subcompartments or microdomains of the plasma membrane are assumed to play important functions in cellular transport processes (transcytosis, potocytosis, endocytosis, and cholesterol flux) and transmembrane signal transduction ("caveolae signaling hypothesis") (79). Morphological appearance of caveolae directly correlates with caveolin expression. Caveolin is expressed in tissue-specific manner with three isoforms (caveolin 1, 2 and 3; see Fig. 8) and two different translation initiation sites (caveolin 1 α and 1 β) (80–82). It functions as a marker, coat and scaffolding protein, based on its capacity to form homo-oligomers and to self-associate into precursor-like "pre-caveolae". The functional domains were defined recently (Fig. 8) (81–84). The biochemical correlate of caveolae can be isolated as so-called detergent-insoluble glycolipid-enriched rafts (DIGs), since they resist solubilization by 1% TX-100

in the cold and are characterized by a low buoyant density, due to their high content of lipids (85). Actually DIGs may correspond to "pre-caveolae" (86).

The putative involvement of caveolin, the GPI-PLC, and GPI lipids/proteins in Amaryl action in adipocytes and their localization in caveolae/DIGs raises the possibility of a direct interaction of Amaryl with these components. Therefore, we studied the putative association of authentic radiolabeled Amaryl with adipocyte membrane fractions by direct photoaffinity labeling (40). Significant photocrosslinking of $[^3H]Amaryl$ to polypeptides of an apparent size between 5 and 250 kDa was not observed. Radiolabeled Amaryl was found covalently bound predominantly to two lipid species of similar migration behaviour (during thin layer chromatography), which were recovered predominantly with DIGs. Low or minute amounts of Amaryl, only, were found associated with lipids from plasma membranes and microsomes. A direct interaction of Amaryl with glycolipids residing in DIGs was suggested by two-dimensional thin layer chromatographic analysis of the photoaffinity-labeled lipids. The migration behaviour of the major Amaryl-labeled material was similar to, but not identical with, free GPI lipids prepared from yeast (40). Preliminary data demonstrated

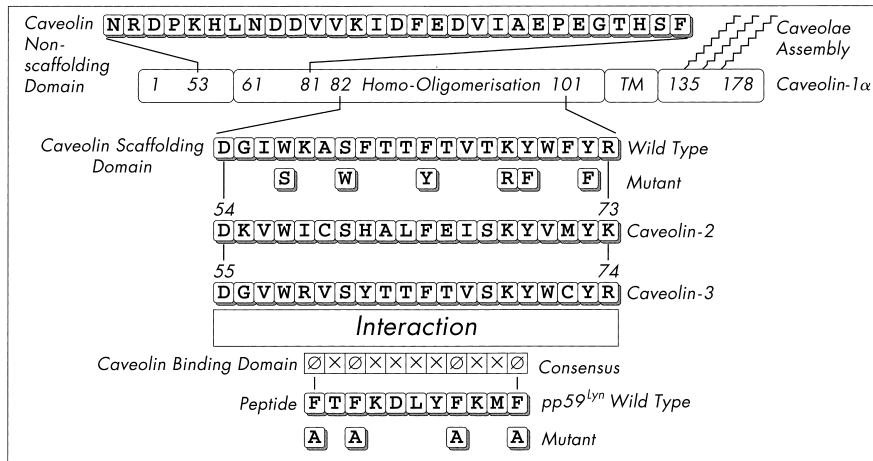


Fig. 8. Domain organization of caveolin. Structures of the (wild type and mutant) caveolin-scaffolding domains of caveolin isoforms 1-3 and (wild type and mutant) caveolin binding domains of pp59^{Lyn}, and the consensus sequence derived for the caveolin binding domain of signaling proteins

are presented (see text for details). The fatty acyl side chains of the carboxy-terminal cytosolic domain of caveolin, which presumably is anchored at the caveolar plasma membrane, are indicated. TM, membrane-interacting region of caveolin.

metabolic labeling of the photocrosslinked lipids with both [¹⁴C]inositol and [¹⁴C]glucosamine compatible with the structure of GPI lipids/anchors. The binding of Amaryl to DIGs was time-dependent. The efficiency of the photolabeling of the two GPI lipid species strongly increased with the incubation time of the adipocytes prior to the irradiation, with steady state reached after 4 to 8 hr. Binding was also non-saturable. The efficiency of the photolabeling increased in a linear fashion over a wide concentration range of [³H]Amaryl present during the incubation and could not be competed for by excess of unlabeled drug (40). Thus, Amaryl seems to intercalate with slow kinetics directly into DIGs of the adipocyte plasma membrane in close vicinity to GPI lipids/anchors, rather than bind to distinct high-affinity (proteinaceous) receptor sites.

As already mentioned, evidence is accumulating that DIGs/caveolae operate as compartments for the regulated assembly of the components for a number of signal transduction cascades (87,88). Lisanti's group previously demonstrated that caveolin interacted via a specific region, the so-called caveolin-scaffolding domain, with a number of signaling proteins, such as the dual-acylated nonreceptor tyrosine kinase pp59^{Lyn}. These signaling proteins were associated with DIGs/caveolae by means of their lipid modification (Fig. 8) (89-91). In fact, it is this interaction between

the caveolin-scaffolding domain and the corresponding caveolin-binding domain of the signaling proteins that keeps them in a basal inactive state, however competent for future activation/signaling (87,88).

Interference of Amaryl with Caveolin Function

We recently showed that introduction of an excess of synthetic caveolin-binding domain peptide into isolated rat adipocytes by electroporation caused dissociation of pp59^{Lyn} from caveolin (G. Müller, S. Welte, S. Wied, W. Frick, manuscript submitted). The apparent interference with the interaction between the caveolin-scaffolding domain of caveolin and the caveolin-binding domain of pp59^{Lyn} (see Fig. 8) correlated well with the concentration-dependent induction of tyrosine phosphorylation of IRS-1. Data from our laboratory now provide strong evidence that sulfonylureas (SU) also can induce dissociation of pp59^{Lyn} and additional caveolar components from caveolin in isolated rat adipocytes (Fig. 9). At concentrations effective for stimulating glucose transport and tyrosine phosphorylation of caveolin, Amaryl, and to a lower degree, glibenclamide, but not tolbutamide, reduced in a concentration-dependent manner the amount of (immunoblotted, IB) pp59^{Lyn} and of the (photoaffinity-labeled) GPI protein, Gce1,

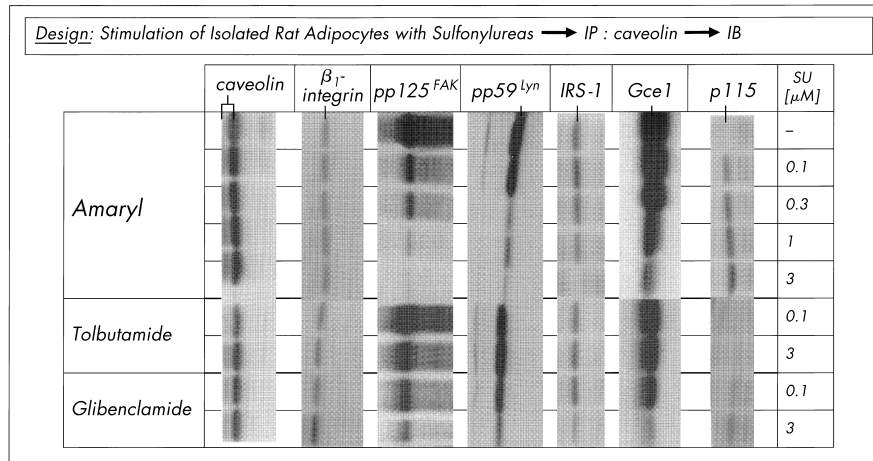


Fig. 9. Effect of sulfonylureas on complex formation between caveolin, pp59^{Lyn}, and cAMP-binding ectoprotein, Gce1. (140; G. Müller, S. Welte, S. Wied, W. Frick, manuscript submitted). Isolated rat adipocytes were stimulated with increasing concentrations of different sulfonylureas for 30 min at 30°C. Total plasma membranes were prepared by differential centrifugation, solubilized and subjected to immunoprecipitation (IP) with anti-caveolin antibodies. The anti-caveolin

immunoprecipitates were immunoblotted (IB; chemiluminescence detection) with specific antibodies for the presence of pp59^{Lyn} and caveolin or photoaffinity-labeled with N₃-[³²P]cAMP for detection of Gce1. Phosphorimages and chemiluminescence images of a typical experiment (repeated three times with similar results) are shown. The homologous immunoblotting with caveolin demonstrated comparable efficiencies of the anti-caveolin immunoprecipitations under the various conditions.

which were co-immunoprecipitated (IP) with caveolin from sulfonylurea-treated adipocytes.

In agreement with a direct effect of Amaryl on the structural and functional organization of caveolae/DIGs, we found that sulfonylureas triggered the redistribution of specific caveolar/DIG components (Fig. 10). To demonstrate this, plasma membranes from isolated rat adipocytes stimulated with different sulfonylureas were fractionated into DIG and non-DIG structures. After purification of the DIGs by sucrose density gradient centrifugation, both fractions were assayed by immunoblotting (IB) or photoaffinity labeling for the presence of a number of caveolar and non-caveolar components. Treatment with each sulfonylurea led to a considerable decrease in the amount of the GPI protein, Gce1, and of pp59^{Lyn} in DIGs, and to a corresponding increase in non-DIG areas of the plasma membrane in a concentration-dependent fashion, with Amaryl being most potent, followed by glibenclamide, gliclazide, and lastly tolbutamide. The amount of caveolin and GLUT4 recovered with DIGs was not affected by sulfonylurea treatment, arguing for the specificity of the redistribution process (Fig. 10). The effect of a long-term treatment with sulfonylureas on the structural organization of caveolae in isolated rat adipocytes was also studied (Fig. 11). 4 hr incubation with

Amaryl and, to a lower degree, with glibenclamide, caused a considerable and concentration-dependent reduction of the amount of pp59^{Lyn} and Gce1. In contrast, the levels of the cytoskeletal proteins, pp125^{FAK} and β_1 -integrin, as well as of caveolin in DIGs prepared from the drug-treated adipocytes, were not altered. This was demonstrated by immunoblotting of total DIG proteins with appropriate antibodies. Tolbutamide had no significant effect on the composition of DIGs (Fig. 11).

Differential Involvement of SUR1/2A in Glibenclamide and Amaryl Action in Adipose, Muscle, and Neuronal Cells

Direct insulin-mimetic and insulin-sensitizing effects of first- and second-generation sulfonylureas on isolated and cultured adipose, and muscle cells, have been described in numerous studies (42–50,92). However, the underlying molecular mechanisms, such as receptors and binding proteins, signal transduction cascades and components, remain ill-defined. For instance, in recent reports, glibenclamide was demonstrated to increase the activities of fatty acid synthase and glycerol-3-phosphate dehydrogenase and to inhibit isoproterenol-stimulated lipolysis

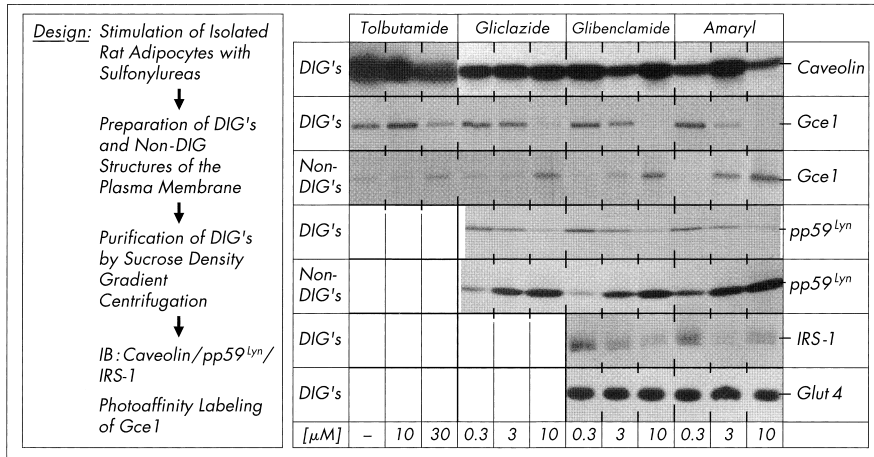


Fig. 10. Effect of sulfonylureas on the distribution of pp59^{Lyn} and cAMP-binding ectoprotein, Gce1, between detergent-insoluble glycolipid-enriched rafts (DIGs) and non-DIG structures of the plasma membrane. (140) Isolated rat adipocytes were stimulated with increasing concentrations of different sulfonylureas for 30 min at 30°C. Plasma membranes were prepared by differential centrifugation and then treated with 1% TX-100 for 1 hr on ice. After centrifugation (100,000X g, 15 min), the detergent-insoluble pellet fraction was subjected to sucrose density gradient centrifugation for purification of DIGs. They were recovered from the 8/20%-interface of the

gradient, collected by centrifugation and then solubilized in sample buffer. The detergent-soluble supernatant fraction (containing the non-DIG structures) was precipitated and then solubilized in sample buffer. Both DIGs and non-DIGs were analyzed by SDS-PAGE and immunoblotting (IB; chemiluminescence detection) for the presence of pp59^{Lyn}, caveolin and glucose transporter isoforms (GLUT4) or photoaffinity labeling with N₃-[³²P]cAMP for the presence of Gce1. Phosphorimages and chemiluminescence images of a typical experiment (repeated twice with similar results) are shown. IRS, insulin receptor substrate.

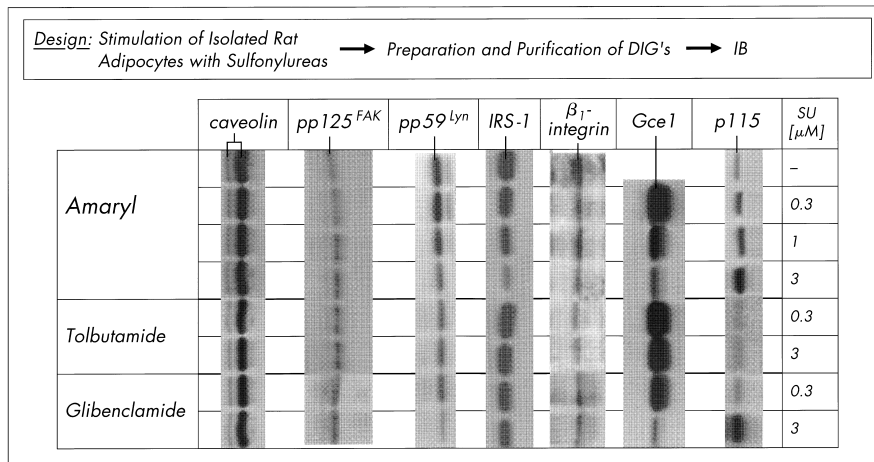


Fig. 11. Effect of sulfonylureas on the protein composition of detergent-insoluble glycolipid-enriched rafts (DIGs). (140; G. Müller, S. Welte, S. Wied, W. Frick, manuscript submitted). Isolated rat adipocytes were incubated with increasing concentrations of different sulfonylureas for 4 hr at 30°C and then lysed by incubation in the presence of 1% TX-100 for 1 hr on ice. After centrifugation (25,000X g, 15 min), the detergent-insoluble pellet fraction was subjected to sucrose density gradient centrifugation. DIGs were recovered from the

8/20%-interface of the gradient, collected by centrifugation (100,000X g, 1 hr), solubilized in sample buffer, separated by SDS-PAGE, and analyzed for the presence of typical caveolar components by immunoblotting (IB; chemiluminescence detection) with specific antibodies. Phosphorimages and chemiluminescence images of a typical experiment (repeated three times with similar results) are shown. IRS, insulin receptor substrate; Gce, cAMP-binding ectoprotein; SU, sulfonylureas.

(93), as well as to potentiate a multitude of typical peripheral insulin effects, such as insulin-stimulated glucose transport, in isolated rat adipocytes (46,48,49). Furthermore, using the isolated perfused rat hindquarter, it was shown that gliclazide alone had an immediate potent stimulatory effect on glucose uptake by skeletal muscle and, together with insulin, had an additive effect (92). These data were confirmed and extended for glibenclamide and the novel sulfonylurea, Amaryl, by our previous studies reviewed here. These results demonstrate a potent direct insulin-mimetic activity of Amaryl, in particular.

In an effort to characterize the molecular mechanism of this peripheral sulfonylurea action, Rajan et al. (94) were unable to identify high affinity sulfonylurea binding sites. Equilibrium binding or specific crosslinking of ^{125}I -labeled 5-iodo-2-hydroxyglibenclamide to the putative SUR in plasma membranes of both isolated rat adipocytes and 3T3-L1 adipocytes was performed under experimental conditions that had been successful in the identification of SUR1 of the pancreatic β cell. Moreover, they were unable to inhibit $^{86}\text{Rb}^+$ efflux (a surrogate parameter for K_{ATP} activity) or to increase cytosolic Ca^{2+} levels with glibenclamide under experimental conditions that were compatible with the demonstration of biological effects of glibenclamide in an insulin-secreting tumor cell line (94). These findings are in agreement with our data of a time-dependent and non-saturable spontaneous insertion of Amaryl molecules directly into caveolae/DIGs of the target cell plasma membrane in close vicinity to GPI lipids, thereby, bypassing the need for a typical high affinity proteinaceous receptor-ligand interaction. This insertion mode of Amaryl action also would explain the apparent discrepancy between the free (not protein-bound) serum drug levels achieved during therapy of NIDDM patients (in the low nM-range) and the free drug concentrations required for induction of significant insulin-mimetic/sensitizing effects in vitro (in the 20- to 300-nM range). This suggests a medium to low affinity interaction of Amaryl with certain target cell structures (see below).

In contrast, several investigators have described specific binding of sulfonylureas in rodent adipocytes and skeletal muscle. For instance, rat adipocyte membranes exhibited specific, saturable glibenclamide-binding ($K_{\text{d}} \sim 1\text{--}3 \mu\text{M}$), which was displaced by other sulfonylureas (49). This

apparent glibenclamide-binding and the functional response to glibenclamide were attributed to the presence of SUR1 in adipose and muscle cells, based on the following experimental evidence: (I) Draznin et al. (95,96) reported that glibenclamide increased cytosolic Ca^{2+} -levels in isolated rat adipocytes in a concentration-dependent manner by promoting Ca^{2+} influx through voltage-dependent Ca^{2+} channels. This effect was blocked by nitrendipine, an L-type Ca^{2+} channel antagonist. (II) Kim, Zemel and coworkers (97,98) demonstrated that cytosolic Ca^{2+} can modulate de novo lipogenesis and lipolysis in a coordinated fashion in both rodent and human adipocytes. (III) In addition, they measured sustained increases in cytosolic Ca^{2+} levels upon glibenclamide challenge (10–20 μM) in human adipocytes, but not in human preadipocytes (93). (IV) The elevation in cytosolic Ca^{2+} correlated nicely with the activation of fatty acid synthase and glycerol-3-phosphate dehydrogenase as well as the inhibition of lipolysis in human adipocytes, with the glibenclamide responses in all these cases completely counteracted by the KCO, diazoxide (93). (V) In extending these findings to the muscle, Pulido and coworkers (92) observed complete reversal of the stimulatory effect of gliclazide on glucose uptake in the perfused rat hindquarter in the presence of diazoxide. (VI) Most strikingly and unexpectedly, Shi and coworkers (93) found apparent expression of SUR1 in human adipocytes, according to both polymerase chain reaction and Northern blots analysis; whereas, human preadipocytes failed to do so. These data combined may be taken as evidence for a role of SUR1 and cytosolic Ca^{2+} in partly mediating or supporting the insulin-mimetic/ sensitizing effects of glibenclamide in adipose and muscle cells. However, unfortunately, demonstration of SUR1 expression at the protein level in adipocytes by photoaffinity labeling with glibenclamide or immunoblotting with antibodies against β -cell SUR1 is still lacking. Furthermore, the described K_{d} - and EC_{50} -values for glibenclamide (in the μM -range) are not compatible with mediation of glibenclamide-binding and effects in adipocytes by high affinity SUR1.

Whatever molecular mechanism peripheral glibenclamide action relies upon, operation of SUR1 and cytosolic Ca^{2+} during Amaryl signaling and action in adipocytes seems unlikely for several reasons. (I) Stimulation of lipogenesis, glycogenesis, glucose transport, GS dephosphorylation, IRS-1 tyrosine phosphorylation,

and pp59^{Lyn} kinase activity in isolated rat adipocytes in response to glibenclamide are reduced by diazoxide to 43–73% and by nitrendipine to 31–45%, confirming the involvement of SUR1/cytosolic Ca²⁺ in glibenclamide action in peripheral cells (Table 1). In contrast, the Amaryl effects are only marginally down-regulated by 8–24% (diazoxide) and 4–16% (nitrendipine). (II) In the presence of diazoxide, both the glibenclamide- and Amaryl-induced elevations of cytosolic Ca²⁺ are reduced dramatically to a similar degree (Table 1). Since the stimulatory effects of glibenclamide and Amaryl on glucose utilization and its regulatory mechanisms are suppressed only partially or not at all, respectively, under conditions that almost completely blocked Ca²⁺ influx and, thereby, SUR1 action, Ca²⁺-independent signaling processes are triggered by glibenclamide and, in particular, Amaryl. (III) Photoaffinity labeling studies with authentic [³H]Amaryl and isolated rat adipocytes

do not result in specific radiolabeling of a 140- to 150-kDa polypeptide corresponding to the size of β -cell SUR1 under experimental conditions, which leads to covalent crosslinking of Amaryl to GPI lipids of adipocyte plasma membrane DIGs (see above). (IV) The β -cell activity, as reflected in binding affinity and depolarization potency of Amaryl, is significantly lower, compared with glibenclamide and most likely based predominantly on SURX, rather than SUR1 (see above). As a consequence, one would expect lower insulin-mimetic/sensitizing signaling by Amaryl vs. glibenclamide, provided the molecular basis for peripheral Amaryl action relies on the same SUR as the Amaryl-induced insulin release from β cells. As already outlined in detail, the opposite is the case, with Amaryl exhibiting a significantly higher capability for insulin-mimetic signaling, direct stimulation of glucose utilization, and insulin-independent blood glucose decrease than glibenclamide.

Table 1. Effect of diazoxide and nitrendipine on the modulation of cytosolic Ca²⁺ levels, glucose metabolism, and signaling by glibenclamide and Amaryl

Effect	% Sulfonylurea-induced effect left in the presence of diazoxide/nitrendipine			
	Glibenclamide Diazoxide	Glibenclamide Nitrendipine	Amaryl Diazoxide	Amaryl Nitrendipine
Lipogenesis	27 ± 8	61 ± 11	80 ± 6	95 ± 6
Glycogenesis	38 ± 11	58 ± 7	79 ± 8	90 ± 6
Glucose Transport	52 ± 14	65 ± 10	89 ± 8	98 ± 8
GS Dephosphorylation	44 ± 9	68 ± 9	81 ± 5	94 ± 7
IRS-1 Phosphorylation	57 ± 10	69 ± 10	85 ± 7	92 ± 10
Caveolin Phosphorylation	35 ± 6	56 ± 9	92 ± 8	96 ± 8
pp59 ^{Lyn} Activation	42 ± 7	55 ± 6	89 ± 6	90 ± 5
Cytosolic Ca ²⁺	6 ± 1	47 ± 7	10 ± 2	41 ± 7

Isolated rat adipocytes prepared as described previously (51) were treated with 10 μ M Amaryl or glibenclamide alone or in combination with 10 μ M diazoxide or 30 μ M nitrendipine for 30 min at 30°C prior to assaying lipogenesis for 90 min (136), glycogenesis for 60 min (42), 2-deoxyglucose transport for 20 min (51), GS dephosphorylation for 15 min (125), IRS-1 and caveolin tyrosine phosphorylation for 10 min (40,137), and cytosolic Ca²⁺ levels for 5 min. Prior to fluorometric Ca²⁺ measurement, the adipocytes were washed three times (by flotation) with Hepes-based salt solution (HBSS); 140 mM NaCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 0.9 mM NaH₂PO₄, 4 mM NaHCO₃, 5 mM glucose, 2 mM sodium pyruvate, 2 mM glutamine, 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 1% bovine serum albumin]. The cells were then loaded with Fura-2-acetoxymethyl ester (10 μ M) in the same buffer for 45 min at 37°C in the dark with continuous shaking. For removal of extracellular dye, the cells were washed three times with HBSS and resuspended in HBSS at 3.5 × 10⁵ cells/ml. Cytosolic Ca²⁺ was determined using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. After the establishment of stable baseline, the response to Amaryl and glibenclamide was determined. Digitonin (25 μ M) and Tris/ethylene glycol tetraammonium acetate (EGTA) (100 mM) were used to measure maximal and minimal fluorescence to calibrate the signals. Cytosolic Ca²⁺ was calculated by the equation of Grynkiewicz et al. (138). The sulfonylurea-induced effect (difference of presence and absence of drug) in the absence of diazoxide and nitrendipine was set at 100% for each parameter for each sulfonylurea. The values represent means ± standard deviation (SD) from (at least) three different adipocyte preparations, each, with determinations in quadruplicate. GS, glycogen synthase; IRS-1, insulin receptor substrate - 1.

The differential effects of diazoxide on the insulin-mimetic activity of glibenclamide and Amaryl, as well as the lack of pronounced insulin-mimetic activity of tolbutamide in fat and muscle cells, is most easily explained by two assumptions. (I) SUR2A, but not SUR1, is involved in the extrapancreatic activity of glibenclamide, based on the high level of expression in fat and adipose tissue, as well as high sensitivity toward KCOs of SUR2A in comparison with SUR1. SUR1 harbors high-affinity binding sites for both sulfonylurea and benzamido moieties (see above); whereas, SUR2A exhibits the benzamido-binding site, only (33,99). Since glibenclamide possesses both sulfonylurea and benzamido moieties, it interacts with SUR1 at two sites (accounting for irreversible inhibition of KIR6.2/SUR1 currents due to rare simultaneous unbinding from both the sulfonylurea- and benzamido-binding sites). However, glibenclamide interacts only with a single site on SUR2A (accounting for rapid unbinding due to dissociation from a single site, only), which may be required and sufficient for initiation of insulin-mimetic effects. The interaction of tolbutamide with SUR1 via its sulfonylurea moiety (accounting for its rapid reversibility on β -cell K_{ATP} currents), but not with SUR2A (due to the missing benzamido moiety), is compatible with the observed negligible insulin-mimetic activity of tolbutamide in peripheral cells. Thus, any (hypothetical) binding of glibenclamide (or Amaryl) to SUR1 in fat and muscle cells would not induce insulin-mimetic signaling. (II) Amaryl binds to SUR2A with lower efficiency, compared with SUR1, due to lack of a typical benzamido moiety limiting its efficiency of insulin-mimetic signaling in peripheral cells via K_{ATP} and Ca^{2+} .

The latter assumption is supported indirectly by the β -cell activity and chemical structure of Amaryl. Amaryl is characterized by a significantly lower binding affinity to and depolarization potency of β -cell membranes than glibenclamide (see above). This is determined by a concerted action between the benzamido- and sulfonylurea-binding sites of SUR1. The benzamido- (meglitinide-), but not the sulfonylurea- (tolbutamide-), moieties differ significantly between Amaryl and glibenclamide (see Fig. 1). Thus, the benzamido moiety of Amaryl seems to contribute less to the overall interaction than that of glibenclamide. Since binding of sulfonylureas to SUR2A is assumed to be

mediated by the benzamido moiety exclusively, one might expect lower affinity of Amaryl for binding to SUR2A, compared with SUR1, as well as compared with glibenclamide.

The experimental findings and considerations mentioned above suggest that if there is some interaction of Amaryl with SUR proteins in cells of extrapancreatic tissues, SUR1 may be preferred relative to SUR2A. A key question in this regard is whether binding of sulfonylureas to K_{ATP} in peripheral tissues has any adverse effects (100,101) and whether a sulfonylurea drug with greater β -cell (i.e. SUR1 vs. SUR2A) specificity (such as possibly Amaryl) would be preferable or would lead to less impairment of ischemic preconditioning, and less predisposition to larger infarctions and dangerous arrhythmias. The largest and most recent study, the UK prospective diabetes study (102), examined whether the incidence of microvascular and macrovascular complications in NIDDM patients subjected to intensive blood glucose control was influenced by the type of therapy. Interestingly, it found no difference in the mortality or diabetic end points of patients treated with insulin, glibenclamide or chlorpropamide. Since glibenclamide binds SUR2A with high affinity; whereas, chlorpropamide (structurally related to tolbutamide) is likely to exhibit greater β -cell specificity, this result suggests that interaction of sulfonylureas with SUR2A does not necessarily influence mortality rates for reasons that remain a matter of speculation (33). In any case, the proposed higher selectivity of Amaryl for SUR1 vs. SUR2A has to be demonstrated by direct means. This has been hampered by the fact that, even for sulfonylureas with a typical benzamido moiety (such as glibenclamide), high-affinity binding to SUR2A has not been possible to show (13,103,104). This may be due to reversible binding of the drug and rapid dissociation from SUR2A when membranes containing the cloned receptors are washed during the binding protocol.

The demonstration of pronounced expression of SUR1 in neuronal cells of the brain, which explains high-affinity binding of glibenclamide to crude cortical membrane preparations (105), raises the possibility of central effects of sulfonylureas. The function of SUR1 and its putative association with K_{ATP} in neurons remains unclear. However, it is known that glucose influences the membrane potential of a subset of glucose-responsive neurons in

the hypothalamus indirectly through its oxidation to generate ATP, which in turn controls the activity of K_{ATP} in the plasma membrane. Closure of K_{ATP} in response to increasing intracellular concentrations of ATP raises the cytosolic level of potassium ions, which depolarizes the neuron and increases its firing rate (106). Interestingly, the glucose-responsive neurons become hyperpolarized and, therefore, decrease their firing rate within minutes after application of leptin (107,108). Leptin is a key hormone for the control of energy homeostasis, which is released by fat cells in direct proportion to cell size (i.e. degree of adiposity) and induces reduction of food intake and increase of energy expenditure (109). The inhibitory effect of leptin on the neuronal firing rate is based in part on its ability to keep the K_{ATP} in the open configuration, thereby, lowering the membrane potential. This can be detected even in isolated patches of plasma membranes excluding a mechanism involving the transcriptional activity of leptin. Although regulation of hypothalamic neuropeptide gene expression by leptin is well described, the leptin signaling of satiety may not rely solely on the Janus kinase-signal transducers and activators of transcription (JAK-STAT) transcriptional pathway (110), but may include also the K_{ATP} nutrition sensing system. According to this possibility, inhibition of K_{ATP} in hypothalamic neurons should interfere with (some aspects of) leptin regulation of energy homeostasis (111).

The well-documented moderate, but continuous, weight gain of NIDDM patients during long-term treatment with conventional sulfonylureas may be based on (partial) antagonism of leptin action at the level of the hypothalamic K_{ATP} , in addition to stimulation of lipogenesis in course of increased pancreatic insulin secretion. Both events may rely on sulfonylurea-binding to SUR1 in hypothalamic neurons and β cells, respectively. Interestingly, smaller increases in body weight of NIDDM patients treated with Amaryl, compared with glibenclamide, have been revealed by preliminary clinical studies (E. Draeger, personal communication). This may be explained by a combination of diminished leptin antagonism and insulin release in response to the lower SUR1-binding and K_{ATP} depolarization activities of Amaryl vs. glibenclamide (see above), provided that SUR1 and KIR6.2/6.1 behave similar in pancreatic β cells and hypothalamic neurons. Interestingly, opening of the K_{ATP} in

response to leptin also has been demonstrated for cultured β cells. Thereby, leptin antagonizes sulfonylurea-induced insulin release in vitro and may interfere with glucose-triggered insulin release in vivo. This raises the possibility that hyperleptinemia (commonly found in obesity) and, as a consequence, inhibition of β cell depolarization is the major mechanism whereby obesity causes NIDDM, at least with regard to β cell failure as the initial causative trigger. The relationships between leptin signaling through K_{ATP} of both hypothalamic neurons and β cells in vivo; its effects on neuronal firing rate, pancreatic insulin release, satiety, and weight control; and the hypothetical interference of sulfonylureas with leptin action at both hypothalamic neurons and β cells, including the molecular mechanism(s) involved [leptin inhibition of K_{ATP} by direct binding to SUR1 (112) or, more likely, by indirect means (113,114)] are important areas for future research.

Molecular Model of Amaryl Action in Peripheral Cells Involving Caveolae/DIGs

According to the experimental data just summarized, Amaryl and glibenclamide support direct glucose utilization by different mechanisms, such as interaction with caveolae/DIGs and SUR2A, respectively. From a structural point of view, this conclusion reinforces the sometimes neglected or even seemingly contradictory possibility that the sulfonylurea moiety, which is shared by both drugs, can adopt considerably different biophysical and biochemical features, depending on the residual portions (i.e. benzamido moiety) of the complete molecule. It will be a challenge to elucidate the structure-activity relationship and to develop a pharmacophore model that covers the distinct modes of interaction of Amaryl and glibenclamide with peripheral cells.

Considering the biochemical studies with isolated and cultured adipose and muscle cells, we propose the following working model for direct insulin-mimetic signaling and metabolic action by Amaryl (Fig. 12). Amaryl partitions directly into caveolae/DIG areas of adipocyte/myocyte plasma membranes in a nonsaturable and time-dependent fashion, thereby, causing redistribution of specific DIG/caveolar components. This may be accomplished by a direct

physical interference of Amaryl with the structure/organisation of DIGs and/or lipolytic release of GPI lipids/proteins from DIGs via Amaryl-induced activation of the GPI-PLC. As a consequence, an acylated nonreceptor tyrosine kinase (non RTK), such as pp59^{Lyn}, dissociates from caveolin and migrates into non-DIG areas of the plasma membrane, thereby, being relieved from inhibition. These events are accompanied/supported by tyrosine phosphorylation of caveolin (40), which may further destabilize the interaction or inhibit the reassociation between pp59^{Lyn} and caveolin. This putative positive feedback loop between caveolin and pp59^{Lyn} for keeping pp59^{Lyn} in the active state is compatible with recent findings that, in 3T3-L1 adipocytes, the homolog of pp59^{Lyn}, pp60^{Fyn}, is a candidate kinase for the insulin-induced tyrosine phosphorylation of caveolin (115). The activated non RTK phosphorylates IRS proteins at specific tyrosine residues and, thereby, initiates metabolic insulin-mimetic signaling to the lipid and glycogen synthesis pathways and the GLUT4 translocation apparatus along the insulin signaling cascade downstream of IRS via the PI-3K pathway (Fig. 12) (57).

Interestingly, a number of extracellular stimuli have been demonstrated to cross-talk to the insulin signal transduction chain via insulin receptor-independent tyrosine phosphorylation of IRS-1 or 2 (116–119). In contrast, mitogenic insulin-mimetic signaling to DNA

synthesis, transcription, and translation via the Ras/mitogen-activated protein kinase (MAPK) pathway presumably involves tyrosine phosphorylation of the Src homology collagen (SHC) proteins; followed by docking of growth factor receptor-bound protein 2 (Grb2); and activation of nucleotide exchange factor son-of-sevenless (SOS), the small G protein, ras, and the kinases, raf, MEK kinase (MEKK), MAPK and extracellular signal related kinase kinase (MEK) and MAPK (see Fig. 12). SHC has been demonstrated to act as substrate for the insulin receptor kinase (57), but may not be accepted by pp59^{Lyn}. The caveolae-dependent mode of insulin-mimetic metabolic signaling critically depends on the presence of a high number of DIGs/caveolae in the target cell, which is certainly true for fully differentiated cells, such as adipocytes and myocytes (78). Other dividing cell types use distinct caveolin-independent mechanisms for regulation of (i.e. keeping silent) non RTKs, thereby, guaranteeing the required degree of specificity.

Strikingly, insulin-mimetic signaling by synthetic phosphoinositolglycans (PIG), which are structurally related to the polar core glycan head groups of GPI lipids [generated by GPI-PLC cleavage; (120–123)], in isolated rat adipocytes has been demonstrated recently to involve tyrosine phosphorylation of IRS-1 by activated pp59^{Lyn} (124–127). PIG favour the metabolic vs. the mitogenic branch of insulin ac-

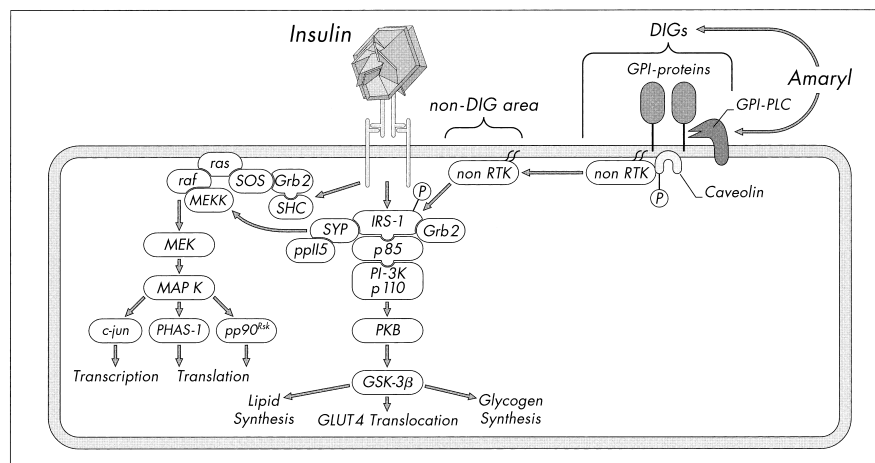


Fig. 12. Hypothetical model for insulin-mimetic metabolic signaling by Amaryl via caveolae/DIGs. (See text for details). DIG, detergent-insoluble glycolipid-enriched rafts; non RTK, dual acylated non-receptor tyrosine kinase; GPI-PLC, glycosyl-phosphatidylinositol-phospholipase C; IRS, insulin receptor substrate; son-of-

sevenless (SOS), MAPK and extracellular signal related kinase kinase (MEK), MAPK, mitogen-activated protein kinase; Grb 2, growth factor receptor-bound protein 2; GSK-3 β , glycogen synthase kinase 3 β GLUT, 4 glucose transporter isoforms; 4 SHC.

tion, as deduced from PIG stimulation of the IRS-1/PI-3K pathway, to almost the full insulin response and of the MAPK pathway to only about 40% of the maximal insulin response (125). Thus, it is tempting to speculate that pp59^{Lyn} and homologous non RTKs are the common key players for the positive cross-talk of various signaling pathways to insulin signaling via tyrosine phosphorylation of IRS-1 and 2, which predominantly leads to metabolic insulin action. The signaling cascades and components responsible for appropriate redistribution/activation of non RTKs, such as pp59^{Lyn}, in response to the various signals may be shared in part or differ completely, as is apparently the case for Amaryl and PIG. We recently identified a 115-kDa polypeptide of the adipocyte plasma membrane that was required for insulin-mimetic metabolic signaling by PIG (88,126), but apparently did not interact with Amaryl (see above). The molecular elucidation of the signaling pathways for Amaryl and PIG upstream of pp59^{Lyn} should reveal novel targets for pharmacological cross-talk to metabolic insulin signaling.

Certainly, the proposed molecular mode of Amaryl action in peripheral cells on basis of the caveolin/pp59^{Lyn} pathway does not exclude the operation of other additional mechanisms. The demonstrated Amaryl-induced increase in GLUT1/4 gene/protein expression (55,56) and diacylglycerol production (70) in conjunction with activation of certain protein kinase C isoforms, which is assumed to mediate GLUT4 translocation in response to insulin at least in part (57), may contribute to and/or enhance the direct insulin-mimetic and/or insulin-sensitizing effect of Amaryl on glucose transport. Furthermore, first- and second-generation sulfonylureas have been shown to affect other systems as well, including: modulating catecholamine release and metabolism; blocking K⁺ channels other than the K_{ATP}; inhibiting transporters, such as multidrug resistance proteins and acidic dopaminic metabolite transporters; increasing cytosolic Ca²⁺ concentrations in K_{ATP}-independent fashion in cells other than the β cells; and forcing development of the aorta. However, these extrapancreatic effects are observed at glibenclamide concentrations in the μ M range only. In the absence of (time-dependent) accumulation of the drug at the corresponding target, as is most likely the case for Amaryl in DIGs/caveolae (see above), and taking into account the therapeutic serum concentrations of Amaryl (see below), these

mechanisms defined *in vitro* presumably do not contribute to the *in vivo* hypoglycemic activity of Amaryl.

The receptor-independent and time-dependent interaction of sulfonylureas with peripheral cells via insertion into DIGs/caveolae apparently is most efficient for Amaryl, compared with conventional sulfonylureas, such as glibenclamide. This may facilitate operation of this mechanism of insulin-mimetic signaling *in vivo*, despite the fact that the total (free and protein-bound) serum concentration of Amaryl measured after once daily oral treatment of NIDDM patients with 4 mg for 7 days approached only about 0.7 μ M at 2.5 hr after the last administration (128–130). This concentration is about one to two orders of magnitude below the total drug levels effective *in vitro* in the presence of 1% bovine serum albumin in the incubation medium (i.e. 10–50 μ M), assuming a similar high-protein binding of Amaryl (about 99.5%) *in vivo* and *in vitro*. Compatible with the proposed time-dependent and functional accumulation of Amaryl molecules in DIGs of the target cell, we observed a considerable leftward shift of the concentration-response curves for Amaryl activation of the GPI-PLC, lipid synthesis, and anti-lipolysis in rat adipocytes upon increasing the incubation period (40,42).

Conclusions

The reviewed pharmacological and biochemical findings lead to the following conclusions on the pancreatic and extrapancreatic activity of sulfonylureas. (I) The binding, depolarisation, and insulin-releasing characteristics of Amaryl and glibenclamide indicate that different sulfonylureas can interact with different SURs of a common pore-forming subunit that constitute the K_{ATP}. (II) Sulfonylureas appear to decrease blood glucose by induction of insulin release and in conjunction with an insulin-independent activity, even after a single oral administration to normal animals. (III) Amaryl exhibits more pronounced insulin-independent blood glucose-lowering activity, compared with conventional sulfonylureas. (IV) This insulin-independent activity can be explained by direct stimulation of glucose transport (via GLUT4 translocation) and non-oxidative glucose metabolism in fat and muscle cells. (V) At the molecular level, Amaryl seems to intercalate spontaneously into caveo-

lae or DIGs of fat and muscle cells, thereby, (a) activating a GPI-PLC, (b) causing redistribution of caveolar components, such as GPI lipids, GPI proteins, and non RTKs, (c) triggering tyrosine phosphorylation of caveolin, and (d) inducing downstream cross-talk to the insulin signaling cascade via insulin receptor-independent tyrosine phosphorylation of IRS-1/2 (see Fig. 12). (VI) The concentrations of Amaryl required for insulin-mimetic signaling events and activation of glucose transport/metabolism in isolated adipose cells are higher than the therapeutic plasma drug levels. (VII) Provided that the observed time-dependent accumulation of Amaryl in caveolae/DIGs of peripheral cells represents the initial trigger for the described cross-talk from caveolae to the insulin signaling cascade and would also occur in vivo, the insulin-independent blood glucose-lowering activity of Amaryl might become effective during therapy, even at lower drug concentrations, due to the longer exposure times compared to in vitro. (VIII) In light of the ongoing discussion of hyperinsulinism and long-lasting blockade of K_{ATP} as a concern in the pathogenesis and therapy of NIDDM including diabetic late complications (e.g. cardiovascular side effects), one may speculate that the higher insulin-independent blood glucose-lowering activity of Amaryl might be of therapeutic relevance.

In summary, our studies provide the following mechanistic explanations for the therapeutic advantages of Amaryl vs. second-generation sulfonylureas, such as glibenclamide. The faster and longer action (131, 132), the lower doses required (133), and the lower insulin levels generated (34,37,38) seem to rely on a combination of reduced insulin secretion and increased direct, as well as insulin-stimulated, glucose utilization in peripheral tissues. The lower cardiovascular risks (134, 135) and atherogenic potential (Y. Satoh and S. Shakuto, manuscript submitted) may be based on higher selectivity for the K_{ATP} of the β cell vs. other tissues. At the molecular level the pharmacological characteristics of Amaryl may be explained in part by its interaction with SURX in the pancreatic β cell and DIGs/caveolae in adipose and muscle cells.

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*Note added in proof

After completion of the revision process, first results from a placebo-controlled, double-blind, cross-over clinical study on Amaryl effects on peripheral insulin sensitivity of healthy, glucose tolerant and insulin resistant offspring of patients with NIDDM were reported (Volk A, Maerker E, Rett K, Häring HU, Overkamp D (2000) Glimepiride—effects on peripheral insulin sensitivity. *Diabetologia* 43 (supplement 1):A39 [abstract]). Acute infusion of Amaryl during a three-step hyperinsulinemic euglycemic glucose clamp increased significantly the metabolic glucose clearance rate at low and moderate insulin levels. Since stimulation of pancreatic insulin secretion was suppressed by somatostatin, Amaryl apparently exerts insulin-sensitizing/mimetic activity in peripheral tissues of relatives of NIDDM patients compatible with the animal and in vitro findings reviewed here.

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(space limitation necessitated restriction of citations to reviews and studies directly related to Amaryl action which provide experimental details, more intense discussions of specific items and additional references)

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