Evidence for a 65 kDa sulfonylurea receptor in rat pancreatic zymogen granule membranes

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Abstract In rat pancreatic zymogen granules (ZG), a K^+ selective conductance which can be blocked by ATP has been characterized. Here we show that this pathway can be specifically blocked by glibenclamide. Using a rapid filtration assay, we also found specific binding of [^{3}H]glibenclamide to a low-affinity site (K_d 5.6 ± 1.1 μM) in rat pancreatic zymogen granule membranes (ZGM). In photoaffinity labeling experiments with [^{3}H]glibenclamide, a 65 ± 1.5 kDa polypeptide was specifically labeled. Previously, a ~65 kDa mdr1 gene product has been demonstrated to be involved in the regulation of the K^+ selective conductance of ZG. We conclude that this protein may be a subunit of, or associated with, a ZG K_{ATP} channel.

Key words: K_{ATP} channel; Sulfonylurea receptor; Glibenclamide; Secretory granule; MDR1

1. Introduction

In zymogen granules (ZG) of the exocrine pancreas, a K^+ conductance has been described which may be involved in enzyme and fluid secretion induced by secretagogues [1]. The K^+ conductance can be blocked by ATP, non-hydrolysable ATP analogues and glibenclamide and activated by diazoxide in the presence of MgATP. These are characteristic features of ATP-sensitive K^+ (K_{ATP}) channels, that have first been found in the heart and in pancreatic β-cells [2,3]. The K_{ATP} channels of the pancreatic β-cell and the heart have been shown to be composed of two different subunits [4]: the channel pore is formed by Kir 6.2, a member of the inwardly rectifying potassium (Kir) channel family, while the other subunit represents the sulfonylurea receptor (SUR) [5,6]. The SUR is a member of the ATP binding cassette (ABC) transporter superfamily [7], which also includes the multidrug resistance (mdr) P-glycoprotein (for review, see [8]) and the cystic fibrosis transmembrane conductance regulator (CFTR) [9].

Previously, we have shown that the K^+ conductance of ZG is composed of a K^+ selective and a non-selective cation conductance [10]. In this study, we show that the K^+ selective but not the non-selective cation conductance can be specifically blocked by glibenclamide. In addition, we describe the characterization of a low-affinity binding site for [^{3}H]glibenclamide in rat pancreatic zymogen granule membranes (ZGM) by a rapid filtration assay and its putative localization on a 65 kDa protein by photoaffinity labeling. This corresponds to the molecular weight of a protein cross-reacting with mdr1 antibodies which is involved in the regulation of the K^+ selective cation conductance in pancreatic ZG [10,11]. The data suggest that the 65 kDa protein photolabeled by glibenclamide is a subunit of the K^+ channel underlying the ATP- and glibenclamide-blockable K^+ conductance present in ZGM.

2. Materials and methods

2.1. Materials

[^{3}H]Glibenclamide (~50 Ci/mmol) was obtained from Dupont NEN (Bad Homburg, Germany). Unlabeled glibenclamide, gliquidone, gliclacide, glipizide and glisoxepide were a kind gift of Dr. H. Englert (Hoechst AG, Frankfurt/Main, Germany). Soluene-350 and Ultima Gold were from Packard (Groningen, The Netherlands). Pefabloc SC was from Boehringer (Mannheim, Germany). Carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine tetraphosphate (AttP), adenosine 5′-(β,γ-methylene)triphosphate (AMP-PCP), guanosine triphosphate (GTP) and bovine serum albumin were from Sigma (Deisenhofen, Germany). Percoll was from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Isolation of ZG and purification of ZG membranes

ZG from rat exocrine pancreas were isolated as described earlier [12,13]. Briefly, pancreatic tissue from male Wistar rats (180–250 g; Charles River Wiga GmbH, Sulzfeld, Germany) was homogenized by nitrogen pressure cavitation (500 psi), and ZG were isolated by centrifugation of the homogenate on a self-forming continuous Percoll-Gradient (40% Percoll v/v at 20 000 × *g* for 20 min). ZG form a distinct dense whitish band on the bottom of the gradient. For separation from minor mitochondrial contaminations ZG were washed in an isotonic buffer containing 50 mM succinate followed by centrifugation at 1000 × *g* for 20 min [14].

For purification of ZGM the protocol described by Thévenod et al. [13] was used with minor modifications. ZG were diluted about 20-fold in a hypotonic 'lysis buffer' containing 10 mM HEPES, 0.1 mM $MgSO_4$, adjusted to pH 7.0 with Tris, and a protease inhibitor 'cocktail' (10 μM leupeptin, 100 μg/ml trypsin inhibitor, 2 mM benzamidine, 0.2 mM Pefabloc SC). During incubation on ice the suspension cleared within 45 min and was then centrifuged for 1 h at 100 000 × *g*. The pellet was resuspended in 'lysis buffer' and centrifugation was repeated. The protein concentration of the final pellet containing the ZGM was determined according to Bradford [15] using bovine serum albumin as a standard. Membranes were stored at −70°C until use.

2.3. Measurement of cation conductance

Cation conductance of pancreatic ZG was assayed according to a previously reported protocol for the quantitative evaluation of macroscopic ion fluxes through endogenous conductance pathways of ZG membranes [1]. This assay relies on the measurement of osmotic lysis of ZG resuspended in buffered isotonic salt solutions, which is induced by addition of electrogenic ionophores allowing for membrane

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Abbreviations: ABC, ATP binding cassette; CCCP, carbonyl-cyanide *m*-chlorophenylhydrazone; CFTR, cystic fibrosis transmembrane conductance regulator; KATP channel, ATP-sensitive K^+ channel; Kir channel, inwardly rectifying K^+ channel; mdr, multidrug resistance P-glycoprotein; SUR, sulfonylurea receptor; ZG, zymogen granules; ZGM, zymogen granule membranes

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permeation of counterions. Granule lysis causes a decrease in absorbance of the suspension which is measured at a wavelength of 540 nm in a Beckman DU-64 spectrophotometer at 37°C.

ZG were suspended in 150 mM K^+ or Na^+ acetate solution containing 1 mM EDTA and buffered with 50 mM imidazole (pH 7.0, adjusted with acetic acid). Since the intragranule pH is about 6.5, and the membrane permeability for protons is low [1], an inside-to-outside directed H^+ concentration gradient of ~0.5 pH units is generated across the granule membrane. Addition of 16 μM of the electrogenic protonophore CCCP, which maximally permeabilizes the granule membrane to H^+, converts the H^+ concentration gradient into an inside negative H^+ diffusion potential which, in turn, energizes cation influx through endogenous cation permeabilities. Anion influx occurs through the uncharged molecule acetic acid, which permeates through the lipid membrane by non-ionic diffusion and dissociates to provide the intragranule space continuously with protons for protonation of imidazole as well as for proton efflux from the acidic interior [1]. Under these conditions cation influx through endogenous cation permeabilities becomes rate-limiting.

Since bulk salt influx into the intragranule space and the resulting granule lysis are limited by the flux of ions through the endogenous conductance pathway, but not by the flux of counterions through the shunt pathway, the slope of the decrease in absorbance with time will represent an estimate of the rate of ions transported through the endogenous conductance pathway. Lysis rates were expressed as inverse half-times of lysis, which were considered proportional to the rate constant of lysis. Half-time of granule lysis was estimated from the slope of the decrease in absorbance with time between addition of ionophore and either experimental half-time or the entire observation period if the half-time was not reached. The slope of the absorbance change with time was estimated by linear regression of the digitized data.

2.4. Rapid filtration assay

A rapid filtration assay for measurement of [^{3}H]glibenclamide equilibrium binding was performed essentially as described by Gaines et al. [16], with minor modifications. Briefly, 100 μg of ZGM were incubated with 10 nM [^{3}H]glibenclamide in assay buffer (50 mM Tris/HCl, 0.1 mM Pefabloc SC, pH 7.4; 250 μl assay volume) in the presence or absence of unlabeled drugs for 1 h at room temperature (varying incubation times between 15 min and 2 h showed that equilibration was complete after 1 h). Stock solutions of unlabeled drugs were prepared in dimethylsulfoxide (DMSO), the final concentration of which in the assay was 1% (at this concentration DMSO did not affect equilibrium binding). All determinations were performed in duplicate. Incubation was terminated by addition of 3.5 ml of ice-cold assay buffer followed by immediate filtration over Whatman GF/F glass fiber filters under reduced pressure. Filters were washed 3 times with 3.5 ml of buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

IC_{50} values were obtained from competition experiments by fitting of the data to the general dose–response equation for a single binding component [17]. According to the Cheng-Prusoff equation, IC_{50} values were equated with K_i values or, in the case of homologous competition, with K_d values [18].

2.5. Photoaffinity labeling

Photoaffinity labeling was performed according to Kramer et al. [19]. 100 μg of ZGM were incubated with 100 nM [^{3}H]glibenclamide in assay buffer (as described under 'rapid filtration assay') in a total volume of 50 μl. Non-specific binding was determined in the presence of 20 μM unlabeled glibenclamide. After equilibration for 1 h at room temperature the samples were transferred to microtiter plates and irradiated with ultraviolet light (254 nm wavelength) at a distance of 5 cm for 8 min on ice (~0.8 J/cm^2). For separation of unbound radioactivity samples were then diluted 10-fold with buffer containing 10 mM Tris/HCl, 4 mM EDTA, 4 mM iodoacetamide, 0.1 mM Pefabloc SC, pH 7.4, and centrifuged for 30 min at 48 000×g. The membrane pellets were resuspended in sample buffer containing 2% SDS and 5% 2-mercaptoethanol (pH 6.8) and separated by SDS-PAGE on 7.5% acrylamide minigels [20]. The lanes were cut into 3 mm pieces which were placed in scintillation vials. After addition of 0.4 ml Soluene-350 for elution of radioactivity from the gel pieces and 8 ml Ultima Gold and incubation overnight, radioactivity was measured in a liquid scintillation counter.

2.6. Statistics

All experiments were performed at least three-fold; the exact number of experiments is given with the respective results. Variability of the data is expressed as means ± S.D. or means ± S.E.M. Statistical analysis of the granule lysis data was done with the Statgraphics program using paired Student's t-test. Non-linear regression analysis of the binding data according to the least-squares method was done with the Sigma-Plot program (Jandel Scientific Software, San Rafael, USA).

3. Results

3.1. Measurement of cation conductances

The presence of an ATP-sensitive, K^+-selective and a non-selective cation pathway in ZG has been described previously [10]. The K^+-selective conductance is inhibited by ATP and its non-hydrolysable analogues [10]. About 60% of the granule lysis in isotonic K^+ buffer is mediated by the K^+-selective conductance, whereas the remaining 40% are mediated by the non-selective cation conductance [10].

To further characterize the K^+-selective conductance in ZG, we compared the inhibitory effect of glibenclamide on the cation conductance of granules incubated in K^+ or Na^+

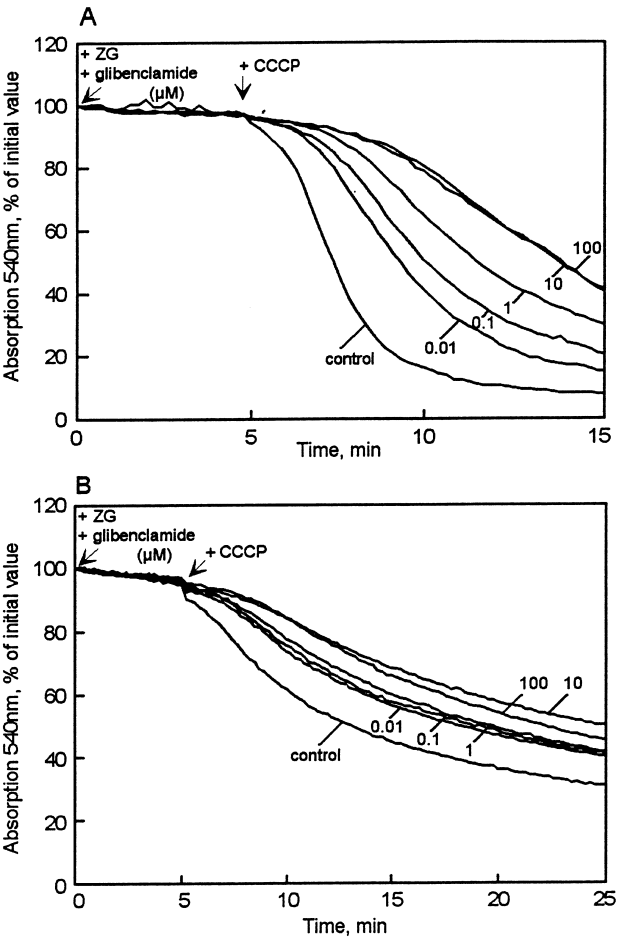

Fig. 1. Effect of glibenclamide on K^+ and Na^+ conductance of zymogen granules (ZG). ZG were incubated in isotonic K^+ acetate (A) or Na^+ acetate (B) imidazole buffer plus or minus the indicated concentrations of glibenclamide in the cuvette at 37°C for 5 min. The absorbance of the ZG suspension was followed at 540 nm. K^+ conductance (A) and Na^+ conductance (B) were measured after addition of the protonophore, CCCP (at the arrow).

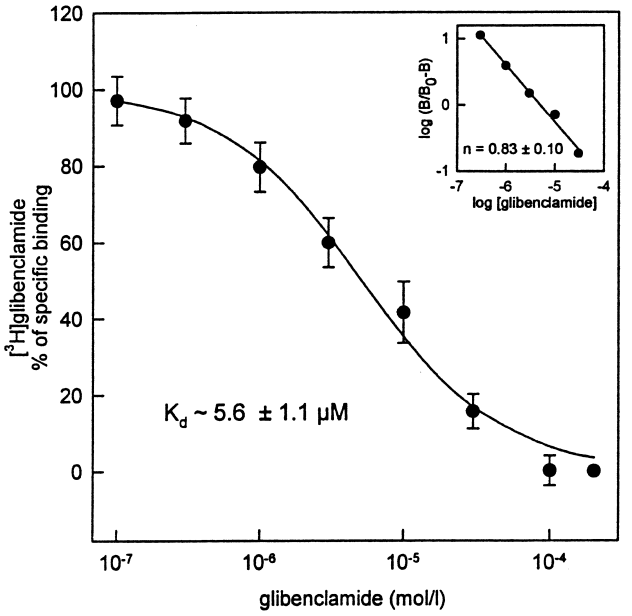

Fig. 2. Binding inhibition of [^{3}H]glibenclamide by glibenclamide in rat pancreatic ZGM and Hill plot of the data (inset). Non-specific binding was defined at 200 μM glibenclamide. 100% specific [^{3}H]glibenclamide binding (total binding–non-specific binding) corresponded to 169 ± 14 dpm (means ± S.E.M.). The curves show pooled data from 8 experiments (± S.E.M.).

acetate buffer. The effect of different concentrations of glibenclamide (10 nM–100 μM) on ZG lysis in K^+ and Na^+ acetate after addition of CCCP is shown in Fig. 1A,B. Glibenclamide inhibits K_{ATP} channels by binding to a high-affinity SUR [5]. An effect of glibenclamide on non-selective cation channels is not described. Dose-dependent inhibition of CCCP-induced ZG lysis with glibenclamide was observed in K^+ acetate (Fig. 1A) but not in Na^+ acetate (Fig. 1B). Maximal inhibition of ZG lysis in K^+ acetate occurred with 10 μM glibenclamide; the control lysis rate of $30.3 \pm 8.9 \cdot h^{-1}$ was reduced by 71.4 ± 5.4% to $8.2 \pm 1.8 \cdot h^{-1}$ (means ± S.D., $n = 9$, $P < 0.0001$, paired Student's t-test). With Na^+ acetate, control lysis rate of ZG was $8.1 \pm 0.6 \cdot h^{-1}$, addition of 10 nM–100 μM glibenclamide reduced ZG lysis independently of the concentration of glibenclamide tested by about 40%. At the maximal concentration of glibenclamide tested (100 μM) lysis was reduced by 42.3 ± 7.2% to $4.7 \pm 0.4 \cdot h^{-1}$ (means ± SD, $n = 3$, $P < 0.02$ paired Student's t-test). This indicates that glibenclamide specifically inhibits the K^+-selective conductance as compared to the Na^+-permeable non-selective cation conductance in ZG.

3.2. Rapid filtration assay

By competition experiments with unlabeled glibenclamide a specific component of [^{3}H]glibenclamide binding to ZGM was detected which accounted for 43 ± 1.6% of total binding. A further characterization of the specific binding component was done by competition with increasing doses of unlabeled glibenclamide. A K_d of 5.6 ± 1.1 μM (means ± S.E.M.; $n = 8$) for glibenclamide binding was determined from the inhibition curves (Fig. 2). The Hill plot yielded a slope factor of 0.83 ± 0.1, indicating a lack of cooperativity in binding (Fig. 2, inset). A B_{max} of 11 ± 2.4 pmol/mg protein was calculated from the binding amplitude, taking into account the degree of saturation at the given label concentration [21]. Scatchard analysis of saturation binding data calculated from the inhibition curves according to Boer et al. [22] confirmed the existence of a single class of low-affinity binding sites (data not shown).

For pharmacological characterization of the glibenclamide binding site in ZGM, sulfonylurea derivatives known to bind to SUR, K_{ATP} channel activators and substances that inhibit the K^+ conductance in ZG [1] were tested for their ability to displace [^{3}H]glibenclamide from ZGM. Of the second-generation sulfonylureas tested, gliquidone inhibited [^{3}H]glibenclamide binding in a similar concentration range to that of glibenclamide with an IC_{50} of ~2 μM. The potency of binding inhibition of the other second-generation sulfonylureas was lower, and the overall sequence gliquidone > glipizide > glisoxepide > gliclacide was determined (Table 1). The first-generation sulfonylurea, tolbutamide, did not inhibit [^{3}H]glibenclamide binding at a concentration of 100 μM, which is in agreement with its lack of inhibition of ZG K^+ conductance [1].

Nucleotides also inhibited [^{3}H]glibenclamide binding to ZGM (Table 1). ATP and the non-hydrolysable ATP analogues, AMP-PCP and AttP, were the most effective nucleotides tested. The inhibition by ATP was independent of Mg^{2+} (data not shown), suggesting that binding inhibition of [^{3}H]glibenclamide by ATP does not involve a phosphorylation-mediated process.

The K^+ channel blockers, quinine and quinidine, which

Table 1
Inhibition of [^{3}H]glibenclamide binding to ZGM by sulfonylureas, nucleotides, K_{ATP} channel openers and blockers of ZG K^+ conductance

Compound	Concentration (M)	Percent of binding inhibition (means ± S.E.M.)	n
Gliquidone	10^{-4}	102 ± 6	3
Glipizide	10^{-4}	65 ± 9	4
Glisoxepide	10^{-4}	49 ± 2	4
Gliclacide	10^{-4}	35 ± 10	4
Tolbutamide	10^{-4}	1 ± 14	3
ATP	5×10^{-4}	62 ± 8	6
AMP-PCP	5×10^{-4}	61 ± 11	5
AttP	5×10^{-4}	64 ± 9	4
ADP	5×10^{-4}	51 ± 5	3
GTP	5×10^{-4}	54 ± 8	3
Minoxidil sulfate	10^{-4}	4 ± 17	5
Diazoxide	10^{-4}	5 ± 9	5
Quinidine	10^{-4}	23 ± 11	5
Quinine	10^{-4}	12 ± 11	4

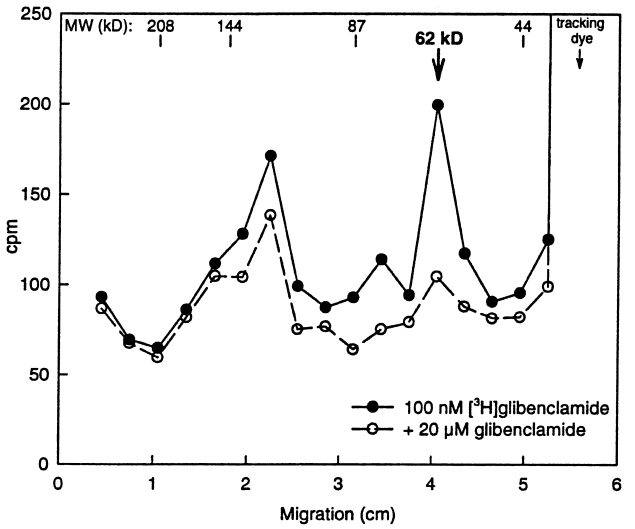

Fig. 3. Photoaffinity labeling of ZGM with [^{3}H]glibenclamide. One out of 4 similar experiments is shown. The experiment was performed as described under Section 2 (x-axis, position of the gel slice; y-axis, radioactivity in cpm eluted from the gel slice; top, positions of the molecular weight markers).

block the K^+ conductance in ZG [1], also inhibited [^{3}H]glibenclamide binding in ZGM. The K_{ATP} channel openers, minoxidil sulfate and diazoxide, had only a small inhibitory effect on [^{3}H]glibenclamide binding at the concentrations tested (Table 1).

3.3. *Photoaffinity labeling*

As glibenclamide is photoactivatable and can be linked to its receptors by UV irradiation [19], photoaffinity labeling experiments were performed for identification of the glibenclamide-binding protein in ZGM. In preliminary experiments, the effects of different wavelengths, irradiation times and [^{3}H]glibenclamide and protein concentrations were examined (data not shown). By an optimized protocol as described under Section 2, a peak of covalently bound radioactivity that could be displaced by unlabeled glibenclamide was obtained at 65 ± 1.5 kDa (means ± S.E.M.; $n = 4$; Fig. 3). This corresponds to the molecular weight of a ~65 kDa mdr1 gene product that is supposed to regulate K^+ and Cl^- conductances in ZG [10,11].

4. Discussion

In this study, we could show that glibenclamide inhibits the K^+ selective cation conductance of ZG completely in a dose-dependent manner. The inhibition of the Na^+-permeable cation conductance was partial and independent of the concentration of glibenclamide tested, suggesting a non-specific effect. Based on these results, we investigated the binding properties of ZGM for [^{3}H]glibenclamide. Using a rapid filtration assay, we found a single class of low-affinity binding sites with a K_d of 5.6 ± 1.1 μM, which in photolabeling experiments could be shown to be associated with a 65 kDa protein.

The main differences between the glibenclamide binding site of ZGM and SUR 1 are the 10^3–10^4-fold higher affinity of SUR 1 for [^{3}H]glibenclamide binding as compared to the ZGM binding site and the different molecular weights (SUR 1 has a predicted molecular weight of 177 kDa) [5]. Furthermore, there are differences with respect to their pharmacological profiles. The rank of potencies of second-generation sulfonylureas for [^{3}H]glibenclamide binding inhibition is glibenclamide > glipizide > gliquidone > glisoxepide > gliclacide for the high-affinity site of pancreatic β-cells [23], while in ZGM the sequence is glibenclamide = gliquidone > glipizide > glisoxepide > gliclacide. Moreover, ATP requires Mg^{2+} for inhibiting [^{3}H]glibenclamide binding to the pancreatic β-cell high-affinity site, and it cannot be replaced by non-hydrolysable ATP analogues [24]. In ZGM, the inhibitory effect of ATP on [^{3}H]glibenclamide binding was independent of Mg^{2+}, and also non-hydrolysable ATP analogues were inhibitory.

Apart from the high-affinity sites probably representing SUR 1, in several tissues, such as pancreatic β-cells, smooth muscle and brain, low-affinity glibenclamide binding sites have been described (K_d between 2 nM and 16 μM; for review, see [25]). Some of these sites may represent SUR 2, an isoform of SUR 1 that has recently been cloned [6]. SUR 2 has a predicted molecular weight of 174 kDa and is assumed to couple to Kir 6.2 to form the cardiac K_{ATP} channel. A detailed pharmacological profile of SUR has not been published. In addition, recently a mitochondrial low-affinity glibenclamide binding site has been associated with a 28 kDa polypeptide, which has been postulated to represent the mitochondrial SUR [26].

Another member of the ABC-transporter superfamily, CFTR, has also been associated with the regulation of K^+ channel activity: CFTR coexpression with Kir 1.1b (ROMK2) enhances the glibenclamide sensitivity of Kir 1.1b [27].

Previously, we have shown that a ~65 kDa mdr1-gene product is involved in the regulation of K^+ and Cl^- conductances in ZG [10,11]. This corresponds to the molecular weight of the glibenclamide binding protein in ZGM as determined by photoaffinity labeling experiments. In combination with a recent preliminary report describing the presence of a Kir channel protein in ZGM [28], our data indicate that the ATP-sensitive K^+ channel of ZGM, similar to the K_{ATP} channel of pancreatic β-cells, may be composed of several subunits, including an ABC transporter.

There are precedents for the intracellular location of glibenclamide binding proteins: more than 90% of the high-affinity glibenclamide binding sites of pancreatic β-cells have been shown to be located intracellularly, in particular also in association with insulin secretory granules [29,30]. The functional significance of this finding is unclear, but it has been speculated that SURs in secretory granules are involved in plasmamembrane voltage-independent insulin secretion induced by sulfonylureas [31]. Another example is the above-mentioned low-affinity glibenclamide binding site in mitochondria [26].

What is the physiological role of the ATP-sensitive K^+ channel of ZGM? Agonist-induced enzyme secretion in permeabilized pancreatic acinar cells depends on the presence of both cytosolic K^+ and Cl^- [32]. The K^+ conductance of ZG is assumed to be involved in exocytosis of the zymogen granule content after fusion with the plasma membrane through active secretion of K^+ and H_2O into the intragranular space, possibly serving 'flushing out' of macromolecular digestive enzymes [1]. However, the signaling pathways and the mechanisms of activation of K^+ conductance during secretagogue-induced enzyme and fluid secretion remain to be fully elucidated.

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