Effect of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic β -cell line and rat cerebral cortex

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1 The effects of blockers and openers of K^+ channels on binding of [3 H]-glibenclamide to microsomes obtained from a pancreatic β -cell line (HIT-T15) or rat cerebral cortex were examined.

2 The blockers quinine, chlorpromazine and thiopentone and the openers cromakalim [($\pm$) 6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol] and minoxidil sulphate did not significantly interact with the sulphonylurea receptor of HIT-cells both at phosphorylating (presence of MgATP) and dephosphorylating (absence of MgATP) conditions.

3 In the absence of MgATP, pinacidil (200–500 μ M) did not significantly displace [3 H]-glibenclamide binding to microsomes from HIT-cells. The displacement of [3 H]-glibenclamide binding was strongly enhanced by MgATP and was due to a decrease in the number of high affinity binding sites for glibenclamide.

4 MgATP enhanced pinacidil-induced inhibition of [3 H]-glibenclamide binding to microsomes from rat cerebral cortex.

5 The effect of MgATP on pinacidil-induced inhibition of [3 H]-glibenclamide binding was maintained after solubilization of the membranes from HIT-cells or rat cerebral cortex.

6 It is concluded that the sulphonylurea receptor is regulated not only by sulphonylureas but also by the K^+ channel openers, diazoxide and pinacidil, and by protein phosphorylation. The binding sites for sulphonylureas and these K^+ channel openers are not identical, but appear to be located at a single protein or at tightly associated proteins.

Keywords: Pinacidil; glibenclamide; MgATP; sulphonylurea receptor; pancreatic β -cells; cerebral cortex

Introduction

Pinacidil is a vasodilator which hyperpolarizes smooth muscle cells via opening of K^+ -channels in the plasma membrane (for reviews Edwards & Weston, 1990; Robertson & Steinberg, 1990). In addition to its effects on smooth muscle cells, pinacidil activates K^+ -channels in ventricular myocytes (Fan *et al.*, 1990), insulin-secreting cells (Garrino *et al.*, 1989; Dunne, 1990) and oocytes (Honoré & Lazdunski, 1991). In these cells the ATP-dependent K^+ -channel (K-ATP-channel) appears to be the major site of action of pinacidil. The K-ATP-channel is blocked by sulphonylureas (for review see Ashcroft & Ashcroft, 1990). In insulin-secreting cells this block initiates a chain of events eventually leading to the release of insulin, whereas opening of K-ATP-channels by diazoxide or pinacidil inhibits insulin release due to hyperpolarization of the cells (Trube *et al.*, 1986; Lebrun *et al.*, 1988; Garrino *et al.*, 1989; Dunne, 1990).

In microsomal membranes from insulin-secreting cells, a high affinity binding site has been identified which represents the sulphonylurea receptor (Geisen *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987; Gaines *et al.*, 1988; Panten *et al.*, 1989; Niki *et al.*, 1989). In the absence of nucleotides, diazoxide did not significantly displace [3 H]-glibenclamide from the sulphonylurea receptor (Schwanstecher *et al.*, 1991). However, in the presence of the Mg complex of ATP (MgATP), [3 H]-glibenclamide binding was decreased and diazoxide-induced displacement of [3 H]-glibenclamide binding was increased. These findings are consistent with the view that sulphonylureas and diazoxide exert their effects by interaction with the same binding site at the sulphonylurea receptor, the binding properties of which are modulated by protein

phosphorylation (Schwanstecher *et al.*, 1991). However, due to the difficulty in dissolving high concentrations of diazoxide, a more detailed analysis of the interaction between sulphonylureas and diazoxide was not feasible.

Pinacidil inhibited [3 H]-glibenclamide binding to cardiac and cortex membranes only at concentrations higher than 100 μ M (Gopalakrishnan *et al.*, 1991; Angel & Bidet, 1991). These experiments were carried out in the absence of nucleotides. Therefore, the structural similarity of pinacidil to diazoxide (Figure 1) and the better solubility of pinacidil led us to test the hypothesis that sulphonylureas and pinacidil compete for the same binding site in the presence of MgATP. The results do not support this hypothesis.

Methods

Cell culture and preparation of microsomes from HIT-cells

HIT cells were grown as monolayers in RPMI 1640 containing 10% foetal bovine serum, 100 nM sodium selenite, 100 μ g ml⁻¹ streptomycin and 100 u ml⁻¹ penicillin G. Cells were fed two times weekly and subcultured once per ten days as described by others (Hill & Boyd, 1984). Microsomes were prepared from monolayers on 94 mm petri dishes (passage 77–90). The plates were cooled on ice, washed twice with phosphate-buffered saline, and then harvested with a cell scraper. The cells were pelleted (50 g, 5 min, 20°C) and used to prepare crude membranes as follows. Cells were resuspended in ice-cold Tris buffer (5 mM, pH 8.0, 100 μ M phenylmethylsulphonyl fluoride), left for 40 min on ice and then homogenized with 15 strokes of a Teflon-glass homogenizer (300 r.p.m.). The homogenate was centrifuged

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Figure 1 Structures of diazoxide (a), pinacidil (b) and cromakalim (c). For diazoxide and pinacidil the structures predominant in tautomeric equilibrium are shown (Wohl, 1970; Petersen *et al.*, 1978). Asterisks indicate dissociating protons ($pK_a = 8.6$ or 10.8 for diazoxide or pinacidil, respectively).

at 100,000 *g* and 4°C for 30 min, rehomogenized in Tris-buffer (50 mM, pH 7.4, 100 μ M phenylmethylsulphonyl fluoride) and centrifuged again at 100,000 *g* and 4°C for 30 min. Microsomes were rehomogenized in ice-cold Tris buffer (50 mM, pH 7.4, 100 μ M phenylmethylsulphonyl fluoride) and immediately frozen in liquid nitrogen until binding studies or solubilization was performed.

Preparation of microsomes from rat cerebral cortex

Male Wistar rats (300–400 g) fed *ad libitum* were stunned, decapitated, the cerebral cortex was prepared on ice and immediately immersed in ice-cold Tris buffer (290 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 μ M phenylmethylsulphonyl fluoride, 10 mM Tris, pH 7.4). The tissue was homogenized in 10 volumes of this buffer with 10 strokes of a teflon-glass homogenizer (300 r.p.m.), and then centrifuged at 600 *g* and 4°C for 15 min. The supernatant was centrifuged at 100,000 *g* and 4°C for 30 min, rehomogenized in Tris-buffer (50 mM, pH 7.4, 100 μ M phenylmethylsulphonyl fluoride) and centrifuged again at 100,000 *g* and 4°C for 30 min. Microsomes were rehomogenized in ice-cold Tris buffer (50 mM, pH 7.4, 100 μ M phenylmethylsulphonyl fluoride) and immediately frozen in liquid nitrogen until binding studies or solubilization was performed.

Measurement of [³H]-glibenclamide binding to microsomes

Measurement of [³H]-glibenclamide (51 Ci mmol⁻¹) binding to microsomes (at 22°C) was performed as previously described (Schwanstecher *et al.*, 1991).

Solubilization of microsomes

Microsomes from HIT-cells or rat cerebral cortex were thawed, rehomogenized and mixed with ice-cold solubilization buffer to give the final concentrations: 10 mg ml⁻¹ microsomal protein; 1% Triton X-100 or 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 0.2% (w/v) phosphatidylcholine (egg); 10% (w/v) glycerole; 115 mM KCl; 1 mM EDTA; 100 μ M phenylmethylsulphonyl

fluoride; 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.4. The solution was gently stirred for 1 h at 4°C and centrifuged at 120,000 *g* and 4°C for 60 min. The supernatant was then collected and stored at -80°C until binding studies were performed. [³H]-glibenclamide binding of the supernatant was not diminished after centrifugation at 120,000 *g* for 4 h or filtration through a Millex GS-filter (0.22 μ m). The protein content of the microsomal suspensions or the detergent extracts was determined according to Peterson (1977) with bovine albumin used as reference. The amounts of solubilized protein were determined by use of controls with appropriate concentrations of detergent.

Measurement of [³H]-glibenclamide binding to the solubilized receptor

The incubation mixture consisted of 0.7 ml Tris-buffer (50 mM, pH 7.4), 100 μ l [³H]-glibenclamide (3 nM), 200 μ l detergent extract (final protein concentration 400 μ g ml⁻¹) and other additions as described in the Results section. Unless stated otherwise, incubations were carried out for 2 h at room temperature and were terminated as described by Glossmann & Ferry (1983) with minor modifications. Incubations were terminated by addition of 1 ml of an ice-cold mixture containing 0.4% (w/v) bovine γ -globulin, 20% (w/v) polyethylene glycol, 0.4% (w/v) Triton X-100 and 50 mM Tris (pH 7.4). The incubation tubes were vortexed immediately after the addition of this mixture and were kept on ice for 10–20 min. Aliquots were filtered (Whatman GF/C filters, 25 mm diameter, soaked in ice-cold wash buffer before use) under vacuum and washed rapidly three times with 4 ml of a Tris-buffer (50 mM Tris, 8% (w/v) polyethylene glycol, pH 7.4). The ³H content of the filters was determined in a liquid scintillation counter after addition of 4 ml of scintillation fluid (Quickscint 402, Zinsser, Frankfurt, Germany) and equilibration for 24 h at room temperature. Associations were started by addition of microsomes or detergent extract. Nonspecific binding was determined by incubations in the additional presence of 100 nM unlabelled glibenclamide. Specific binding was determined by subtracting nonspecific from total binding.

Treatment of results

Data analysis was performed as previously described (Schwanstecher *et al.*, 1991). Results are presented as mean $\pm$ s.e. mean for independent experiments using separate membrane preparations. Significances were calculated by the two-tailed U-test of Wilcoxon and of Mann and Whitney with the Bonferroni correction for multiple comparisons (Wallenstein *et al.*, 1980). $P < 0.05$ was considered significant.

Relations between drug concentrations and specific binding were analyzed by fitting the function

$$B = 100 \times \left(1 - \frac{[A]^n}{[IC_{50}]^n + [A]^n} \right)$$

or

$$B = B_1 \times \left(1 - \frac{[A]}{[IC_{50(1)}] + [A]} \right) + B_2 \times \left(1 - \frac{[A]}{[IC_{50(2)}] + [A]} \right)$$

to the experimental data by a non-linear least squares routine. B is specific binding, B₁ or B₂ the amount of binding sites for high or low affinity binding, respectively, [A] the drug concentration, IC₅₀ the half-maximally inhibitory concentration (one-site model), IC₅₀₍₁₎ or IC₅₀₍₂₎ the half-maximally inhibitory concentration for high or low affinity binding, respectively (two-site model), n the slope parameter (Hill coefficient). The improvement of the fit assuming a model with two binding sites instead of one binding site was tested by an F-test. The model for two binding sites was

considered to be appropriate when the fit was significantly better ($P < 0.01$) than the one for the single binding site. In no case could the fit be improved by assuming more than two sites. K_D and K'_D values were calculated from half-maximally displacing concentrations (IC_{50}) according to Cheng & Prusoff (1973).

Solutions

Concentrations of the Mg^{2+} -complex of nucleotide and free Mg^{2+} are indicated in the Results section. The free Mg^{2+} concentration was held close to 1 mM by adding appropriate amounts of $MgCl_2$ to the nucleotide containing solutions. The concentrations of free ATP or ADP amounted to 1.9 or 27% of total concentrations, respectively. The required amounts of $MgCl_2$ and the composition of the solutions were calculated for pH 7.4 with a computer programme (Fabiato, 1988). The logarithms of the absolute stability constants ($\log K$) for binding of H^+ and Mg^{2+} to ATP and ADP were taken from Sigel *et al.* (1987) or Taqui Khan & Martell (1962), respectively. The $\log K$ values were corrected for the ionic strength of the incubation buffer (40 mM) (Harrison & Bers, 1987). The $\log K$ values for all proton-ligand complexes were corrected by 0.08 in order to include the proton activity coefficient appropriate for the incubation solution (Harrison & Bers, 1989).

Materials

[3H]-glibenclamide (51 Ci $mmol^{-1}$, 96% radiochemical purity) was from NEN (Dreieich, Germany). Minoxidil sulphate was from Upjohn Co. (Kalamazoo, MI, U.S.A.). Cromakalim [($\pm$)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol] was from Smith-Kline-Beecham (Harlow, GB). Pinacidil was from Leo Pharmaceutical Products (Ballerup, Denmark). Thiopentone was from Byk Gulden (Konstanz, Germany). Chlorpromazine, quinine, polyethylene glycol and phosphatidylcholine (egg) were obtained from Sigma (St. Louis, MO, U.S.A.). Bovine γ -globulin was obtained from Serva (Heidelberg, Germany). Triton X-100 and CHAPS were obtained from Boehringer (Mannheim, Germany). All other chemicals and drugs were obtained from the sources described elsewhere (Schwantescher *et al.*, 1991; Panten *et al.*, 1989).

Results

Table 1 shows that the K^+ channel blockers, quinine (20–500 μM), chlorpromazine (10–100 μM), thiopentone (100–200 μM) and the K^+ channel openers, cromakalim (200 μM) and minoxidil sulphate (100–500 μM), did not significantly alter [3H]-glibenclamide binding to microsomes from HIT-cells in the absence or presence of MgATP (300 μM). In the absence of MgATP pinacidil concentrations up to 500 μM did not significantly displace [3H]-glibenclamide binding to microsomes from HIT-cells (Table 1, Figure 2). Pinacidil, 1 mM displaced [3H]-glibenclamide binding slightly by $15 \pm 2\%$ ($n = 4$, $P < 0.05$; Figure 2). This displacement of [3H]-glibenclamide binding was strongly enhanced by MgATP (Table 1, Figure 2). In the presence of MgATP (300 μM) pinacidil displaced [3H]-glibenclamide binding to microsomes from HIT-cells half-maximally at $490 \pm 25 \mu M$ (Hill coefficient = 0.83; Figure 2). The competitive inhibition assays with unlabelled glibenclamide revealed high ($IC_{50(1)} = 0.88$ nM, $B_1 = 91\%$) and low ($IC_{50(2)} = 0.6 \mu M$, $B_2 = 9\%$) affinity binding of glibenclamide to microsomes from HIT-cells (Figure 2). The $IC_{50(1)}$ value corresponded to a dissociation constant of 0.58 nM (Cheng & Prusoff, 1973). MgATP (300 μM) inhibited binding of [3H]-glibenclamide (0.3 nM) to HIT-cell microsomes by $35 \pm 4\%$ ($n = 4$).

As shown in Figure 3, pinacidil did not increase the half-maximal inhibitory concentration (IC_{50}) for displacement of [3H]-glibenclamide binding by unlabelled glibenclamide in the presence of MgATP. Analysis of these data (Figure 3) assuming a model with two binding sites revealed the following values for $IC_{50(1)}$, B_1 , $IC_{50(2)}$ and B_2 : 1.5 nM, 90%, 1.2 μM , 10% (0 mM pinacidil); 1.4 nM, 62%, 0.7 μM , 9% (0.2 mM pinacidil); 1.5 nM, 36%, 1.2 μM , 6% (1 mM pinacidil). From these experiments the following apparent dissociation constants (K'_D) for high affinity binding of glibenclamide were calculated (Cheng & Prusoff, 1973): 1.2 nM (0 mM pinacidil), 1.1 nM (0.2 mM pinacidil), 1.2 nM (1 mM pinacidil). Calculation of B_{max} values from these data according to DeBlasi *et al.* (1989) indicated that 0.2 or 1 mM pinacidil reduced the B_{max} value for high affinity binding to $63 \pm 3\%$ ($n = 4$, $P < 0.05$) or $36 \pm 4\%$ of the B_{max} value in the absence of pinacidil, respectively.

Table 2 shows that pinacidil-induced displacement of [3H]-glibenclamide binding to microsomes from HIT-cells was not

Table 1 Effects of potassium channel modulators on binding of [3H]-glibenclamide to microsomes from HIT-cells

Drug	Concentration (μM)	[3H]-glibenclamide binding (%)	
		no MgATP	MgATP
Pinacidil	200	98 ± 2	71 ± 2^a
	500	89 ± 3	47 ± 2^a
Cromakalim	200	97 ± 7	98 ± 4
Minoxidil sulphate	100	100 ± 3	97 ± 2
	500	91 ± 3	88 ± 7
Quinine	20	97 ± 5	98 ± 3
	100	105 ± 3	106 ± 2
	500	106 ± 3	102 ± 4
Chlorpromazine	10	106 ± 3	112 ± 6
	100	109 ± 7	100 ± 4
Thiopentone	100	95 ± 3	89 ± 5
	200	87 ± 3	83 ± 6

[3H]-glibenclamide binding was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [3H]-glibenclamide, 1 mM free Mg^{2+} , the indicated concentration of test-drug and no MgATP or 300 μM MgATP. Incubations were started by addition of microsomal protein from HIT-cells (final concentration 200 μg ml^{-1}) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding measured in parallel control incubations (test-drug-free media with or without 300 μM MgATP). The listed values are means $\pm$ s.e.mean for results from 4 separate experiments using separate microsomal preparations.

^a $P < 0.05$ for comparison with the corresponding value in the absence of MgATP.

Figure 2 Effect of pinacidil on binding of [^3H]-glibenclamide to microsomes from HIT-cells (closed symbols) or rat cerebral cortex (open symbols). Inhibition of [^3H]-glibenclamide binding by unlabelled glibenclamide (circles), pinacidil (squares) or pinacidil in the presence of MgATP (triangles) was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^3H]-glibenclamide, 1 mM free Mg^{2+} , the indicated concentrations of displacing drug (logarithmic scale) and no MgATP (circles and squares) or 300 μM MgATP (triangles). Incubations were started by addition of microsomal protein from HIT-cells or rat cerebral cortex (200 or 400 $\mu\text{g ml}^{-1}$ final concentration, respectively) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding of [^3H]-glibenclamide in the absence of unlabelled drugs and in the absence or presence of MgATP. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations. * $P < 0.05$ for comparison with the corresponding value for inhibition of [^3H]-glibenclamide binding by pinacidil in the absence of MgATP.

Figure 3 Effect of pinacidil on the apparent dissociation constant (K_D) for glibenclamide in microsomes from HIT-cells. Inhibition of [^3H]-glibenclamide binding by unlabelled glibenclamide in the presence of 0 (●), 0.2 mM (○) or 1 mM (■) pinacidil was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^3H]-glibenclamide, 300 μM MgATP, 1 mM free Mg^{2+} and the indicated concentrations of unlabelled glibenclamide (logarithmic scale). Incubations were started by addition of microsomal protein (200 $\mu\text{g ml}^{-1}$ final concentration) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding of [^3H]-glibenclamide in the absence of pinacidil and unlabelled glibenclamide. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations.

Table 2 Effect of hexokinase and glucose on pinacidil-induced displacement of [^3H]-glibenclamide binding to microsomes from HIT-cells exposed to MgADP

Nucleotide	[^3H]-glibenclamide binding (%)			
	No HK No Gluc	+ HK + Gluc	+ HK + Gluc	+ HK + Gluc
None	100	98 $\pm$ 3	89 $\pm$ 3	95 $\pm$ 5
MgADP	40 $\pm$ 3 ^a	47 $\pm$ 4 ^a	43 $\pm$ 5 ^a	89 $\pm$ 5 ^b

Incubations in media containing (final concentrations) 1 mM pinacidil, 0.3 nM [^3H]-glibenclamide, 1 mM free Mg^{2+} and the indicated additions [0.5 mM MgADP; 15 μM hexokinase (HK); 20 mM glucose (Gluc)] were started by addition of microsomal protein (200 $\mu\text{g ml}^{-1}$ final concentration) and were terminated after 60 min by filtration. Each single experiment consisted of 8 parallel incubations in different media as indicated. Results are given as percentages of specific binding in the absence of nucleotide, HK and Gluc. All listed values are means $\pm$ s.e. mean for results of 4 separate experiments using separate microsomal preparations. Pinacidil (1 mM) inhibited [^3H]-glibenclamide binding in the absence of MgADP by 24%.

^a $P < 0.05$ for comparison with the corresponding value in the absence of MgADP.

^b $P < 0.05$ for comparison with specific binding in the presence of MgADP and HK (but absence of Gluc).

only enhanced by MgATP but also by MgADP. However, this effect of MgADP was not observed in the simultaneous presence of hexokinase and glucose (Table 2). This finding indicates that the effect of MgADP requires the formation of MgATP.

To characterize further the mechanism by which pinacidil interacts with the sulphonylurea receptor, the receptor was solubilized from HIT-cell microsomes using nonionic (Triton X-100) or zwitterionic (CHAPS) detergents. In the absence of both MgATP and pinacidil, competitive inhibition assay with unlabelled glibenclamide indicated only high affinity binding of glibenclamide to sites solubilized with Triton X-100 from HIT-cells (Figure 4). Analysis of these data (one-site model) revealed a half-maximal inhibitory concentration of 0.72 nM (corresponding to a K_D of 0.42 nM) and a Hill coefficient of 0.96. Addition of MgATP (300 μM) inhibited binding of [^3H]-glibenclamide (0.3 nM) to solubilized binding sites from HIT-cells by 77 $\pm$ 4% ($n = 4$, Triton-extract) or 65% ($n = 2$, CHAPS-extract). In the absence of MgATP, 200 μM pinacidil inhibited [^3H]-glibenclamide (0.3 nM) binding to sites solubilized with Triton X-100 (Figure 4) or CHAPS (results not shown in a figure) by 14 $\pm$ 5% ($n = 4$, $P > 0.05$) or 5% ($n = 2$), respectively. In the presence of MgATP (300 μM), however, 200 μM pinacidil decreased specific binding of [^3H]-glibenclamide (0.3 nM) by 57 $\pm$ 6% (Triton-extract; Figure 4) or 31% ($n = 2$, CHAPS-extract; results not shown in a figure), respectively. In the presence of MgATP (300 μM) pinacidil displaced [^3H]-glibenclamide binding to solubilized binding sites from HIT-cells half-maximally at 140 $\pm$ 8 μM (Hill coefficient = 0.60, Triton-extract; Figure 4) or 790 μM ($n = 2$, CHAPS-extract; results not shown in a figure).

High affinity binding sites for sulphonylureas similar to those in insulin-secreting cells have been found in mammalian brain. To test whether pinacidil-induced inhibition of [^3H]-glibenclamide binding to cerebral sites, was also enhanced by MgATP, membrane-bound and solubilized binding sites were examined. The following values were obtained for inhibition of [^3H]-glibenclamide binding to cerebral sites by unlabelled glibenclamide: $\text{IC}_{50(1)} = 0.52$ nM, $B_1 = 90\%$, $\text{IC}_{50(2)} = 0.9$ μM , $B_2 = 10\%$ (microsomal binding site; Figure

Figure 4 Effect of pinacidil on binding of [3 H]-glibenclamide to solubilized binding sites from HIT-cells (closed symbols) or rat cerebral cortex (open symbols). Inhibition of [3 H]-glibenclamide binding by unlabelled glibenclamide (circles), pinacidil (squares) or pinacidil in the presence of MgATP (triangles) was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [3 H]-glibenclamide, 1 mM free Mg^{2+} , the indicated concentrations of displacing drug (logarithmic scale) and no MgATP (circles and squares) or 300 μ M MgATP (triangles). Incubations were started by addition of solubilized protein (400 μ g ml $^{-1}$ final concentration) and terminated after 120 min by polyethylene glycol precipitation. Results are presented as percentages of specific binding of [3 H]-glibenclamide in the absence of unlabelled drugs and in the absence or presence of mgATP. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations. * $P < 0.05$ for comparison with the corresponding value for inhibition of [3 H]-glibenclamide binding by pinacidil in the absence of MgATP.

2); $IC_{50} = 0.81$ nM, Hill coefficient = 0.96 (Triton-extract; Figure 4, dashed line). The IC_{50} values for high affinity binding corresponded to dissociation constants of 0.22 nM (microsomal site) or 0.51 nM (Triton-extract). Addition of MgATP (300 μ M) inhibited binding of [3 H]-glibenclamide (0.3 nM) to cerebral sites by $38 \pm 3\%$ ($n = 4$, microsomes) or $81 \pm 4\%$ ($n = 4$, Triton-extract). MgATP (300 μ M) significantly enhanced inhibition of [3 H]-glibenclamide binding to microsomes from rat cerebral cortex by 1 mM pinacidil from $10 \pm 2\%$ to $42 \pm 3\%$ ($n = 4$, $P < 0.05$; Figure 2). In the presence of MgATP (300 μ M) pinacidil displaced [3 H]-glibenclamide binding to sites solubilized with Triton X-100 half-maximally at 56 ± 6 μ M (Hill coefficient = 0.61; Figure 4).

Discussion

In the present studies, it was found that blockers of the K-ATP-channel which are structurally unrelated to sulphonylureas (quinine, chlorpromazine, thiopentone) do not significantly interact with the sulphonylurea receptor both in phosphorylating and dephosphorylating conditions. The tested concentrations of quinine (20–500 μ M), chlorpromazine (10–100 μ M) and thiopentone (100–200 μ M) inhibit the K-ATP-channels in insulin-secreting cells almost completely (Bokvist *et al.*, 1990; Kozlowski & Ashford, 1991; Müller *et al.*, 1991). Evidence was previously presented that MgATP-induced inhibition of binding reflects a general phenomenon, being valid for the interaction of all sulphonylureas with their receptor in pancreatic islets (Schwanstecher *et al.*, 1992). This effect of MgATP is accompanied by enhanced displacement of [3 H]-glibenclamide from the sulphonylurea receptor by diazoxide (Schwanstecher *et al.*,

1991). We now show that MgATP also enhances pinacidil-induced displacement of [3 H]-glibenclamide from the sulphonylurea receptor in insulin-secreting cells. Similar effects are not observed for cromakalim (200 μ M) or minoxidil sulphate (100–500 μ M) which have been suggested to open K^+ -channels in smooth muscle or insulin-secreting cells (Edwards & Weston, 1990; Robertson & Steinberg, 1990; Newgreen *et al.*, 1990; Dunne *et al.*, 1990). These findings support the idea that MgATP-dependent interaction with sulphonylurea binding is characteristic of K^+ channel openers structurally related to diazoxide and pinacidil.

Pinacidil-induced displacement of [3 H]-glibenclamide from the sulphonylurea receptor in insulin-secreting cells was not only enhanced by MgATP but also by MgADP (Table 2). However, this effect of MgADP probably requires formation of MgATP by transphosphorylation reactions in the microsomal incubations. This is suggested by the finding that MgADP does not enhance pinacidil-induced displacement of [3 H]-glibenclamide binding when the MgATP concentration is kept low by the hexokinase reaction (Table 2).

In the presence of MgATP, pinacidil decreases the number of binding sites but not the affinity for binding of glibenclamide to its receptor (Figure 3). This observation shows that the inhibitory effect of pinacidil cannot be explained by a competitive interaction of pinacidil and sulphonylureas at the high affinity binding site. Functional studies with vascular smooth muscle also argue against a competitive antagonism between pinacidil and sulphonylureas (Steinberg *et al.*, 1991). The present results do not allow a definite conclusion on the mechanism of the inhibitory effect of pinacidil. However, irreversible binding of pinacidil to the binding site for sulphonylureas is unlikely, since pinacidil-induced opening of the K-ATP-channels in insulin-secreting cells is reversible within 2 min (Garrino *et al.*, 1989). One possible explanation for the interaction between pinacidil and sulphonylureas could be that binding of pinacidil to a distinct receptor allosterically decreases the number of sulphonylurea receptors. In analogy to the suggestion for the morphine-enkephalin receptors (Rothman & Westfall, 1982), pinacidil could allosterically cause K'_D values for sulphonylureas of such magnitude that sulphonylurea binding to the pinacidil-induced conformation of the sulphonylurea receptor would not be detectable.

The inhibitory effect of pinacidil on glibenclamide binding is maintained after solubilization of the sulphonylurea receptor with nonionic (Triton X-100) and zwitterionic (CHAPS) detergents, which differ in their efficiency in preventing aggregation of solubilized proteins. This finding suggests a location of the pinacidil and sulphonylurea binding sites at the same protein or at closely associated proteins.

High affinity binding sites for sulphonylureas similar to those of insulin-secreting cells have been shown to occur in brain (Kaubisch *et al.*, 1982; Geisen *et al.*, 1985; Lupo & Bataille, 1987; Bernardi *et al.*, 1988; Mourre *et al.*, 1990; Gehlert *et al.*, 1990; Treherne & Ashford, 1991; Zini *et al.*, 1991; Angel & Bidet, 1991; Gopalakrishnan *et al.*, 1991). The present study confirms that pinacidil concentrations higher than 100 μ M are required for significant inhibition of [3 H]-glibenclamide binding to brain membranes incubated in the absence of MgATP (Angel & Bidet, 1991; Gopalakrishnan *et al.*, 1991). In high affinity binding sites from cerebral cortex, MgATP caused an enhancement of pinacidil-induced displacement of [3 H]-glibenclamide binding, similar to that described for the sulphonylurea receptor in insulin-secreting cells (Figures 2 and 4). This observation supports the view that the cerebral site represents a sulphonylurea receptor resembling the pancreatic receptor.

The dissociation constants for high and low affinity binding of glibenclamide to membranes from HIT-cells and cerebral cortex are in accordance with those found in earlier investigations (Kaubisch *et al.*, 1982; Geisen *et al.*, 1985; Lupo & Bataille, 1987; Aguilar-Bryan *et al.*, 1990; Zini *et al.*, 1991). However, low affinity binding is missing in detergent

extracts from HIT-cell or brain microsomes (Figure 4). This might be due to selective denaturation of binding proteins with low affinity during solubilization in the presence of Triton X-100. Alternatively, it is conceivable that low affinity binding reflects binding of glibenclamide to phospholipids in the microsomal membranes (Deleers & Malaisse, 1984). After solubilization these phospholipids probably escape precipitation by polyethylene glycol.

In pancreatic β -cells, the potency of diazoxide for activation of K^+ -channels and inhibition of insulin release is about 10 fold higher than the potency of pinacidil (Garrino *et al.*, 1989). In microsomal membranes from pancreatic islets incubated in the presence of MgATP, 140 μ M diazoxide reduced [3 H]-glibenclamide binding by 50% (Schwanstecher *et al.*, 1991). Inhibition by 50% of [3 H]-glibenclamide binding to HIT-cell microsomes was caused by 490 μ M pinacidil in the presence of MgATP (this study). The more pronounced difference between the potencies of diazoxide and pinacidil in intact β -cells as compared to microsomal membranes might be due to destruction of the normal environment for the membrane receptors when preparing microsomes. This view is supported by the increase in potency of pinacidil induced by solubilization of the membrane proteins.

The total concentration of cytosolic ATP in islet cells ranges above 0.5 mM (Malaisse & Sener, 1987) and more than 90% of this concentration probably exists as MgATP complex (Corkey *et al.*, 1986). Therefore, the states of the binding sites for pinacidil and sulphonylureas observed in the presence of MgATP might predominate in the intact β -cell. Even in the presence of MgATP the pinacidil concentration giving 50% displacement of [3 H]-glibenclamide binding from HIT-cell microsomes is high (490 μ M). However, hyperpolarization of the pancreatic β -cells is induced by opening of much less than 10% of all K-ATP-channels in the plasma membrane (Cook *et al.*, 1988; Panten *et al.*, 1990). Hence occupation of a small percentage of the pinacidil receptors would be sufficient for inhibition of insulin release.

Inhibition of the opening activity of the K-ATP-channel in β -cells by ATP does not require the presence of Mg^{2+} (Ashcroft & Ashcroft, 1990). Therefore, MgATP appears to modulate the binding properties of the sulphonylurea receptor via a site not identical with the site mediating ATP-induced channel closure. Moreover, sulphonylureas do not compete with free ATP or MgATP for binding to β -cell microsomes (Schwanstecher *et al.*, 1992). These findings are in favour of the view that MgATP-induced modulation of binding, ATP-induced channel closure and sulphonylurea-induced channel closure are mediated by three separate binding sites. The present study suggests an additional binding site for the K^+ channel openers, diazoxide and pinacidil. The binding sites for these K^+ channel openers on the one hand and for sulphonylureas on the other hand seem to be located at a single protein or at tightly associated proteins. The Mg complex of ADP (MgADP) in the β -cell cytosol increases the opening activity of the K-ATP-channel by interaction with a site probably not identical with the sites mediating ATP- or sulphonylurea-induced channel inhibition (Ashcroft & Ashcroft, 1990; Schwanstecher *et al.*, 1992). In the presence of MgATP, pinacidil (this study) and diazoxide (Schwanstecher *et al.*, 1991), but not free ADP + MgADP (Schwanstecher *et al.*, 1992), diminished [3 H]-glibenclamide binding. Hence, a completely separate binding site for MgADP might exist. It is therefore suggested that channel-opening and channel-closing ligands control the K-ATP-channel via four separate binding sites. A protein kinase tightly associated with the K-ATP-channel and/or regulatory proteins appears to exert additional control. MgATP serves as substrate for protein phosphorylation which could modulate the binding properties of the sulphonylurea receptor.

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