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# CONTROL OF INSULIN SECRETION BY SULFONYLUREAS, MEGLITINIDE AND DIAZOXIDE IN RELATION TO THEIR BINDING TO THE SULFONYLUREA RECEPTOR IN PANCREATIC ISLETS

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**Abstract**—Sulfonylureas inhibit an ATP-dependent $K^+$ channel in the B-cell plasma membrane and thereby initiate insulin release. Diazoxide opens this channel and inhibits insulin release. In mouse pancreatic islets, we have explored whether other targets for these drugs must be postulated to explain their hypo- or hyperglycaemic properties. At non-saturating drug concentrations the rates of increase in insulin secretion declined in the order tolbutamide = meglitinide > glipizide > glibenclamide. The same rank order was observed when comparing the rates of disappearance of insulin-releasing and $K^+$ channel-blocking effects. The different kinetics of response depend on the lipid solubility of the drugs, which controls their penetration into the intracellular space. Allowing for the different kinetics, the same maximum secretory rates were caused by saturating concentrations of tolbutamide, meglitinide, glipizide and glibenclamide. A close correlation between insulin-releasing and $K^+$ channel-blocking potencies of these drugs was observed. The relative potencies of tolbutamide, meglitinide, glipizide and glibenclamide corresponded well to their relative affinities for binding to islet-cell membranes, suggesting that the binding site represents the sulfonylurea receptor. The biphasic time-course of dissociation of glibenclamide binding indicates a complex receptor–drug interaction. For diazoxide there was no correlation between affinity of binding to the sulfonylurea receptor and potency of inhibition of insulin secretion. Thus, opening or closing of the ATP-dependent $K^+$ channel by diazoxide or sulfonylureas, respectively, appears to be due to interaction with different binding sites in the B-cell plasma membrane. The free concentrations of tolbutamide, glipizide, glibenclamide and diazoxide which are effective on B-cells are in the range of therapeutic plasma concentrations of the free drugs. It is concluded that the hypo- and hyperglycaemic effects of these drugs result from changing the permeability of the ATP-dependent $K^+$ channel in the B-cell plasma membrane.

Sulfonylureas block ATP-dependent $K^+$ channels in the plasma membrane of the pancreatic B-cell [1–3]. The resultant depolarization initiates a chain of events eventually leading to the release of insulin [4]. Thus, a specific target has been found which seems to explain the antidiabetic property of sulfonylureas. However, the view that the ATP-regulated $K^+$ channel is not the sole site mediating the hypoglycaemic action of sulfonylureas cannot be readily dismissed. Stimulation of insulin secretion has been reported to require much higher concentrations of glipizide or glibenclamide than $K^+$ channel blockade [3, 5]. Unlike other sulfonylureas, glibenclamide accumulates in B-cells [4, 6], its effects on B-cells appear to be irreversible *in vitro* [7] and an intracellular receptor has been suggested [6]. In addition, *in vitro* effects of sulfonylureas observed in extrapancreatic tissues have been proposed to play a role in their antidiabetic action [8]. However, attributing therapeutic relevance to *in vitro* findings requires these effects to occur at concentrations of free sulfonylureas observed in the plasma from treated patients. Previous studies did not duly consider protein binding of the drugs [8] or protein binding was calculated using inadequate binding constants [5].

The aim of the present investigation was to analyze whether other targets for sulfonylureas must be postulated in addition to the ATP-dependent $K^+$-channel. Therefore, we first measured the free concentrations of tolbutamide, meglitinide (HB 699), glipizide and glibenclamide in our media which contain albumin to prevent binding of insulin to the perifusion system used in secretory studies. Then we examined the concentration–response relationships for the insulin-releasing effects in isolated pancreatic islets in comparison with the free drug concentrations blocking the ATP-dependent $K^+$ channels in the B-cell plasma membrane and the free plasma concentrations achieved under therapy. Furthermore, we studied the reversibility of the insulin-releasing effects of tolbutamide, meglitinide, glipizide and glibenclamide. Finally the binding of these drugs to islet-cell membranes was determined and compared with the biological activities of the drugs since data published previously for insulin-secreting tumor cells

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† Some results of this study were obtained during medical thesis work.

showed considerable variability [5, 9, 10] and might not be representative for B-cells in pancreatic islets. Diazoxide was included in this investigation since it was reported to inhibit insulin secretion by opening the same $K^+$ channel which is closed by sulfonylureas [2, 3].

## MATERIALS AND METHODS

*Chemicals and media.* The following substances were used: octanol (analytical grade) from Aldrich (Milwaukee, WI); crystalline rat insulin from Novo (Bagsvaerd, Denmark); [$^{125}$I]labelled porcine insulin from Behringwerke (Frankfurt, F.R.G.); bovine serum albumin (fraction V, pH 7.0, product number 81003) from Miles (Frankfurt, F.R.G); sucrose and bovine serum γ-globulin (fraction II) from Serva (Heidelberg, F.R.G.); ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), Brilliant Blue R and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) from Sigma (St. Louis, MO); minimal essential culture medium (MEM), horse serum, collagenase (from *Clostridium histolyticum*) and ATP (disodium salt) from Boehringer (Mannheim, F.R.G.); [$^3$H]glibenclamide (53 Ci/mmol, radiochemical purity was 99%), glibenclamide, meglitinide (HB 699) and tolbutamide from Hoechst (Frankfurt, F.R.G.); glipizide from Pfizer (Karlsruhe, F.R.G.); tolazamide from Upjohn (Kalamazoo, MI); gliquidone from Thomae (Biberach, F.R.G.); diazoxide from Essex Pharma (Munich, F.R.G.); penicillin and streptomycin from Biochrom (Berlin, F.R.G.). All other chemicals were analytical grade from Merck (Darmstadt, F.R.G.).

Basal medium for isolation and perifusion of pancreatic islets contained 2 mg/ml albumin and was prepared as previously described [11]. For electrophysiological recording the bath solution on the extracellular side of the cell (containing 2 mg/ml albumin) and the solution for filling the pipette (containing 0.3 mM ATP, free [$Ca^{2+}$] buffered to 50 nM) were prepared as detailed in [3]. Stock solutions of sulfonylureas, meglitinide or diazoxide were prepared daily in NaOH (50 mM).

*Determination of partition coefficients.* Partition coefficients were measured in the octanol-buffer system as outlined by Leo *et al.* [12]. The buffer consisted of 50 mM potassium phosphate and 50 mM boric acid, pH adjusted to 6.3–13 with KOH. The organic phase was saturated with buffer and the water phase was saturated with octanol before partitioning was commenced. Drugs were dissolved in the organic or buffer phase to give a 50 μM concentration. Three-millilitre portions of both phases were mixed and shaken in a mechanical shaker for 2 hr at room temperature (22°), after which the mixture was centrifuged for 30 min at 5000 rpm and 25°. Drug concentration in the appropriate phase was determined before and after partitioning by UV spectrophotometry against the appropriate blank. From the measured apparent partition coefficients and the aqueous pH and $pK_a$ values the partition coefficients for the unionized compounds were calculated [13].

*Determination of albumin binding.* Binding of drugs to albumin was measured by ultrafiltration using the Amicon micropartition system (Type MPS-1 with YMT membrane, Amicon, Witten, F.R.G.). The capped system containing 1 ml of medium was centrifuged in a fixed-angle rotor for 2.5 min at 22° or 37°. The speed was adjusted to give about 300 μl of ultrafiltrate. Before ultrafiltration, media were incubated for 1 hr at room temperature (22°) or 37°. By preincubating the systems and pipettes and by pipetting in an incubator the media were kept at 37° during transfer to the systems. [$^3$H]Glibenclamide was used and the [$^3$H] content in media and ultrafiltrates was measured by liquid scintillation counting. The other drugs were determined in the ultrafiltrates by UV spectrophotometry against appropriate blanks. Ultrafiltrates prepared by parallel centrifugation of albumin-free media containing known drug concentrations served as standards. Binding of drugs to the systems was negligible. An empirical two-constant equation [14] led us to describe the relation between total and free drug concentration by the binding equation $F(C) = b_1 \cdot \ln(b_2 \cdot C + 1) + b_3$. $F(C)$ is the free drug (per cent of total concentration $C$) and $b_1$, $b_2$ and $b_3$ are constants. Curve fitting to the experimental data was accomplished with the NLIN procedure of SAS [15] using the Marquardt method. The calculated constants were used to extrapolate the curves into the range of concentrations which could not be measured by UV spectrophotometry.

*Isolation of pancreatic islets.* Male albino mice (NMRI, 11–15 weeks old, fed *ad libitum*) or ob/ob (obese–hyperglycaemic) mice (non-inbred, 6–8 months old, deprived of food for 44–48 hr) of either sex were used. Pancreatic islets were isolated by collagenase digestion [16] in basal medium supplemented with glucose (5 mM).

*Culture of pancreatic B-cells.* Albino mouse islets were dissociated into single cells by shaking in a solution without $Ca^{2+}$ [16]. The cells were cultured as described in [17] with minor modifications. They were plated for 1–2 days on Nunc Petri dishes (35 × 10 mm, Nunc, Roskilde, Denmark) in minimal essential culture medium (MEM) containing Earle's salt, 5 mM glucose, 10% heat-inactivated horse serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

*Measurement of insulin secretion.* Batches of 50 albino mouse islets were perifused at 0.9 ml/min and 37° as detailed previously [18]. Unless indicated otherwise in the figure legends, the experiments began by perifusing the islets for 60 min with control medium containing 10 mM D-glucose. Switching the distribution valve of the system started medium change and was taken for start of the test period (zero time). No correction for the dead space (about 0.4 ml) between valve and islets on the filter of the perifusion chamber was made. Insulin content of 1–4 min fractions was determined by radioimmunoassay with rat insulin as a reference [19]. Secretory rates were given as percentages of rates at min −2 (100%) and were drawn in the middle of the sampling intervals.

*Electrophysiological recording.* Cultured B-cells were preincubated for 1–2 hr in the presence of albu-

min (2 mg/ml) and glipizide (10 or 100 nM) or glibenclamide (6 nM). By placing a Teflon ring in the culture dish a recording chamber with a volume of 0.6 ml was obtained. The whole-cell configuration of the patch-clamp technique was employed [20] using a set-up previously described [3]. Pipettes were pulled from borosilicate glass and had resistances between 4 and 7 MΩ. The cell membrane potential was held at the resting potential of −70 to −80 mV and hyper- and depolarizing voltage pulses of 10 mV amplitude and 200 msec duration were applied alternatively every 2 sec. This pulse protocol has been established by Trube *et al.* [2] to record currents flowing through ATP-dependent $K^+$ channels in the whole-cell configuration. The current and voltage signals were stored on magnetic tape or filtered by a 4-pole Bessel filter at 110 Hz (−3dB point) and displayed on an oscilloscope or on a chart recorder. Traces shown were photographed from the recorder paper. Outward currents flowing from the cell interior to the bath are indicated by upward deflections and inward currents by downward deflections. Perfusion of the recording chamber at 2 ml/min allowed exchange of the bath solution within about 30 sec. All experiments were performed at room temperature (22°).

*Measurement of* [$^3$H]*glibenclamide binding to microsomes.* About 3000 ob/ob mouse islets were homogenized at 0° in 0.4 ml of medium (200 mM D-mannitol, 65 mM sucrose, 10 mM Hepes, adjusted to pH 7.4 with KOH) using a Potter–Elvehjem homogenizer with a Teflon pestle (10 strokes at 500 rpm). The suspension was transferred to 0.7 ml polypropylene tubes, sonicated for 10 sec as described in [21] and centrifuged at 300 *g* and 4° for 15 min. The supernatant was centrifuged at 70,000 *g* and 4° for 30 min. The microsomal pellet was resuspended in incubation buffer (50 mM Tris–HCl, pH 7.4) and stored frozen at −70° for 1–2 days. [$^3$H]Glibenclamide binding to microsomes was measured at room temperature (22°) in incubation buffer supplemented with [$^3$H]glibenclamide. Associations were started by addition of the microsomes and dissociations by addition of unlabelled glibenclamide (1 μM). Incubations were terminated by rapid filtration of aliquots through Whatman GF/B filters (25 mm diameter, wetted with ice-cold incubation buffer before use) under reduced pressure. Then the filters were immediately washed three times with 4 ml of ice-cold incubation buffer. Filtration and washing took less than 15 sec. The [$^3$H]content of the filters was counted in a liquid scintillation counter after addition of 4 ml of scintillation fluid (Quickszint 402, Zinsser, Frankfurt, F.R.G.) and equilibration for 24 hr at 4°. Nonspecific binding was determined by incubations in the presence of 1 μM unlabelled glibenclamide. Specific binding was determined by subtracting nonspecific from total binding. The protein content of the microsomal suspensions was measured according to McKnight [22].

*Data analysis.* Results of secretion studies are presented as mean ± SE for independent experiments. Significances were calculated by the two-tailed U-test of Wilcoxon and of Mann and Whitney. $P < 0.05$ was considered significant. Relations between drug concentrations and effects or specific binding were analyzed by fitting the function

$$E = \frac{E_{max} \cdot [A]^n}{[EC_{50}]^n + [A]^n} + k,$$

to the experimental data by a non-linear least-squares routine. $E$ is the effect or the specific binding, $E_{max}$ the asymptotic parameter, $[A]$ the drug concentration, $EC_{50}$ the half-maximally effective concentration, $n$ the slope parameter (Hill coefficient), k an additive constant (k = 0 in binding experiments). Analysis of the kinetics of dissociation of [$^3$H]glibenclamide binding was performed by fitting the function $B = B_1 \cdot e^{-k_1 \cdot t} + B_2 \cdot e^{-k_2 \cdot t}$ to the corresponding binding data by a non-linear least-squares routine. $B$ is the total ligand specifically bound at time $t$, $B_1$ and $B_2$ are components of ligand bound at equilibrium and $k_1$ and $k_2$ are dissociation rate constants.

## RESULTS

### *Lipophilicity of hypo- and hyperglycaemic drugs*

To characterize the lipophilicity of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide, the partition of the compounds between octanol and an aqueous phase was measured (Fig. 1). The results for diazoxide were in agreement with other reports [27, 28], whereas the partition coefficient for unionized glibenclamide was lower than published previously [26]. At pH 7.4 the observed apparent partition coefficients for tolbutamide, meglitinide and glipizide were similar (2.9, 2.7 or 2.8, respectively), whereas the calculated value for glibenclamide was 94.

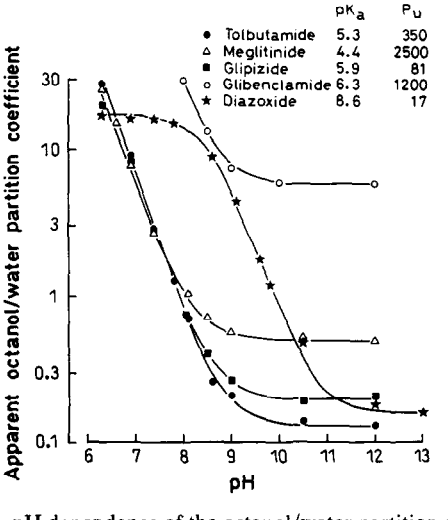


Fig. 1. pH dependence of the octanol/water partition coefficients (logarithmic scale) of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide. Values in the curves are means of results from 2 to 4 separate experiments performed at room temperature. The listed $pK_a$ values in water published previously [23–27], the measured partition coefficients for the dissociated compounds and the apparent partition coefficients measured at aqueous pH values lower than 9 were used to calculate the listed partition coefficients for the unionized compounds ($P_u$).

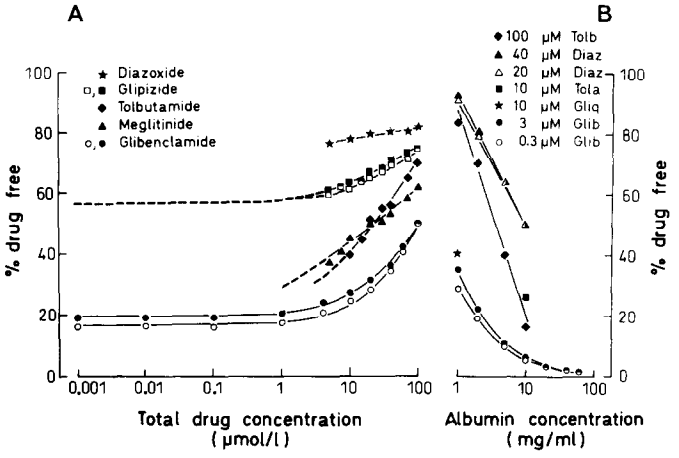


Fig. 2. Binding of sulfonylureas, meglitinide and diazoxide to bovine serum albumin in basal medium. Basal medium is a modified Krebs bicarbonate buffer containing 20 mM bicarbonate and 10 mM Hepes, pH adjusted to 7.4 [11]. (A) The percentages of drug free at different total drug concentrations (logarithmic scale) in the presence of 2 mg/ml albumin were measured at room temperature (open symbols) or at 37° (filled symbols). Values in the curves are means of results from 2 to 5 separate experiments. Curve fitting and extrapolation of the curves (dashed parts) were performed as described in the Materials and Methods section. (B) The percentages of drug free at different albumin concentrations (logarithmic scale) in the presence of the indicated total drug concentrations were measured at 37° (tolbutamide, Tolb; diazoxide, Diaz; tolazamide, Tola; gliquidone, Gliq; glibenclamide, Glib). Values in the curves are means of results from 2 separate experiments. Curves were fitted by eye.

*Albumin binding of hypo- and hyperglycaemic drugs*

Investigation of drug binding to the albumin in our basal medium revealed that in the range of concentrations effective *in vitro*, 19, 56 or 77–81% of glibenclamide, glipizide or diazoxide, respectively, were free at 37° (Fig. 2A). However, binding of tolbutamide and meglitinide was strongly dependent on the total concentrations. Lowering the medium temperature to 22° decreased the free concentration of glibenclamide to 16%, but only insignificantly that of glipizide (Fig. 2A). In the presence of diazoxide (100 μM, total concentration) 17% of glibenclamide (4, 20 or 100 nM, total concentrations) was free in our basal medium at 37°. Figure 2B shows binding data for some sulfonylureas and diazoxide at different albumin concentrations. These data were used to interpret findings in the literature.

*Effects of hypo- and hyperglycaemic drugs on insulin secretion*

In perifused mouse islets both tolbutamide and meglitinide stimulated a steep increase in insulin release reaching a peak rate at min 1.5–3.5, then declining rapidly within the next few min and slowly from min 18 to 58 (Fig. 3). This secretory pattern was seen in each single experiment with the indicated tolbutamide or meglitinide concentrations. However, in the case of glipizide this profile was observed only at the highest concentration tested (1 μM; Fig. 3). At 100, 20, 10, 6 or 3 nM of glipizide half-life of increase in insulin release was about 1.3, 5, 8, 10 or 18 min, respectively, when considering an initial lag of 30 sec and the kinetic of insulin secretion in the absence of drug (Fig. 3). The kinetic of the secretory response to glibenclamide was also concentration-dependent (Fig. 4A). Half-life of increase in insulin release was about 1, 5, 16, 28, 50 or 75 min at 1000, 100, 20, 10, 6 or 3 nM of glibenclamide, respectively. In the presence of the three highest concentrations of glibenclamide the secretory profiles were similar from min 62 to 126. At around min 80 or 106 these secretory profiles were crossed by the traces representing the responses to 10 or 6 nM of glibenclamide, respectively. Thereafter, the latter traces reached comparable plateaus (Fig. 4A) indicating that 10 nM glibenclamide was maximally effective at min 126. At this time insulin release induced by 3 nM of glibenclamide reached its plateau phase and was significantly smaller ($P < 0.05$) than the release in response to 6 nM.

Half-maximally effective concentrations ($EC_{50}$) of free tolbutamide or free meglitinide were 5.1 or 1.7 μM respectively, at min 2.5 (curve fittings not shown) and 5.2 or 1.6 μM, respectively, at min 58 (Fig. 5). Thus, the potency of these drugs did not depend on their length of action. However, the $EC_{50}$ value for free glipizide was 13.1 nM at min 10 (curve fitting not shown) and 4.4 nM at min 58 (Fig. 5). The traces characterized by open symbols in Fig. 7B and perifusions for 2 hr in the presence of 3 and 6 nM of total glipizide (results not shown) demonstrated constant secretory rates around min 118. From these results an $EC_{50}$ value of 4.0 nM was calculated for free glipizide at min 118. The $EC_{50}$ value for free glibenclamide was 15.7 nM at min 10 (curve fitting not shown) and 0.54 nM at min 126 (Fig. 5). When calculating the latter value the maximally effective total concentrations exceeding 10 nM were excluded because they induced secretory profiles not comparable to the kinetics of insulin release at lower glibenclamide concentrations. At min 58 saturating concentrations of tolbutamide, meglitinide, glipizide or glibenclamide caused maximum secretory rates

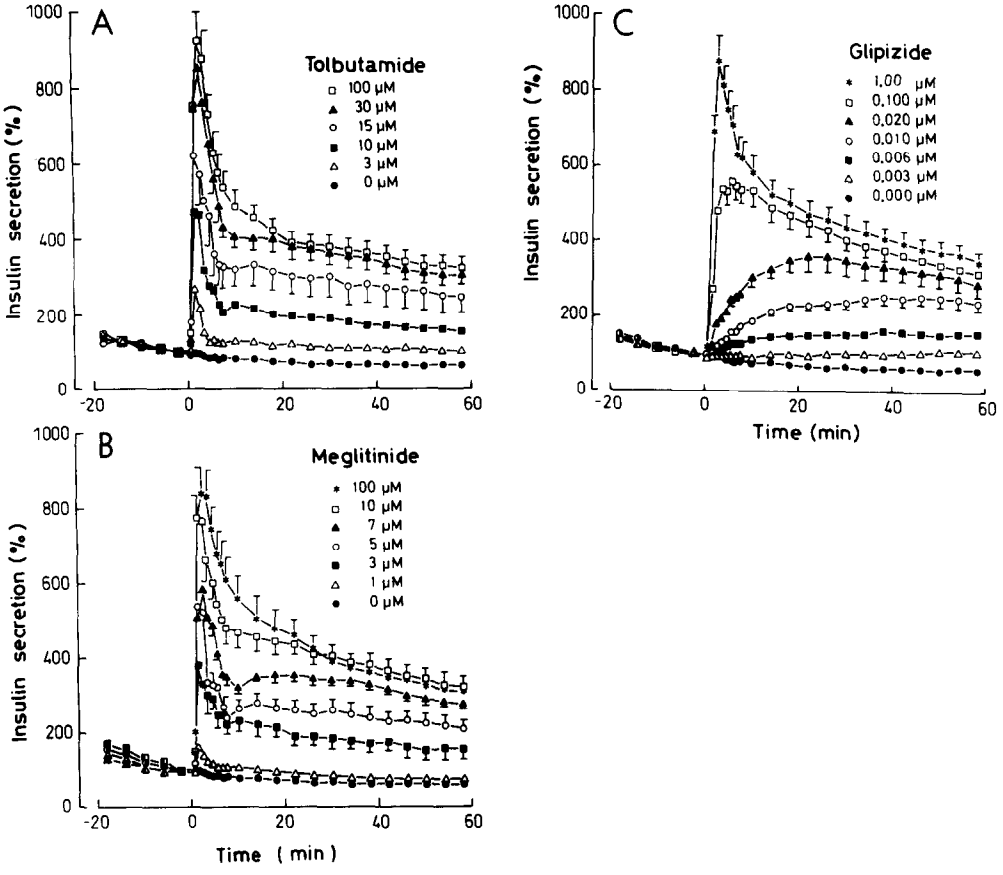


Fig. 3. Concentration-dependent effects of tolbutamide, meglitinide and glipizide on the kinetic of insulin secretion by mouse pancreatic islets. All control and test media contained 10 mM D-glucose. From zero time to min 60 the test media contained the indicated total drug concentrations. Values in the curves are means ± SE of results from 6 to 16 separate experiments.

which did not differ significantly ($P > 0.05$; Figs 3 and 4A).

Insulin secretion induced by a maximally effective glucose concentration (40 mM) was inhibited by 50% in the presence of 11.3 or 11.0 µM of free diazoxide 10 or 58 min after the supply of drug, respectively. These values were derived from the experiments described in Figs 2 and 6 as detailed in the Materials and Methods section (curve fittings not shown). A few minutes after administration of diazoxide new steady states of insulin secretion were established (Fig. 6). However, reversal of inhibition took up to 40 min in accordance with previous observations [29].

The secretory responses to tolbutamide (30 µM) or meglitinide (7 µM) were completely reversible within 10 min (Fig. 7A). However, after removal of an intermediate concentration (10 nM) of glipizide, half-life of decrease of insulin release was 10 min (Fig. 7B). This half-life was prolonged to about 20 min when studying the reversal of the secretory response to a maximally effective concentration (100 nM) of glipizide (Fig. 7B). Rate of insulin secretion in islets perifused for 60 min with D-glucose (10 mM) and glipizide (100 nM, total concentration) decreased from 327 ± 12% at min 58 to 161 ± 5%, 96 ± 3% or 39 ± 3% at min 63.5, 70 or 118, respectively, after switching at min 60 to a medium devoid of glipizide but containing diazoxide (100 µM, total concentration) in addition to 10 mM D-glucose (results from 6 experiments, not shown in a figure). After exposure of islets for 60 min to 20 nM of glibenclamide and subsequent removal of the drug, the secretory profile did not differ significantly from the profile observed in the continuous presence of the same drug concentration (Fig. 8A). Application of this glibenclamide concentration for only 10 or 20 min caused submaximal secretory responses (Fig. 4B). With the beginning of the wash-out of glibenclamide the secretory rates slightly increased for a few min in each single experiment and subsequently there was no indication of reversibility of glibenclamide-induced insulin release within 106–116 min (Fig. 4B). These findings led us to expose pancreatic islets to a high concentration of diazoxide (100 µM) when washing out glibenclamide (Fig. 8B). Under these conditions the rate of insulin secretion decreased within 60 min to one fourth of the rate of controls. Diazoxide (100 µM) was slightly inhibitory in the presence of a 20 nM concentration (Fig. 8B) but not in the presence of a 100 nM concentration of glibenclamide (secretory profiles not shown).

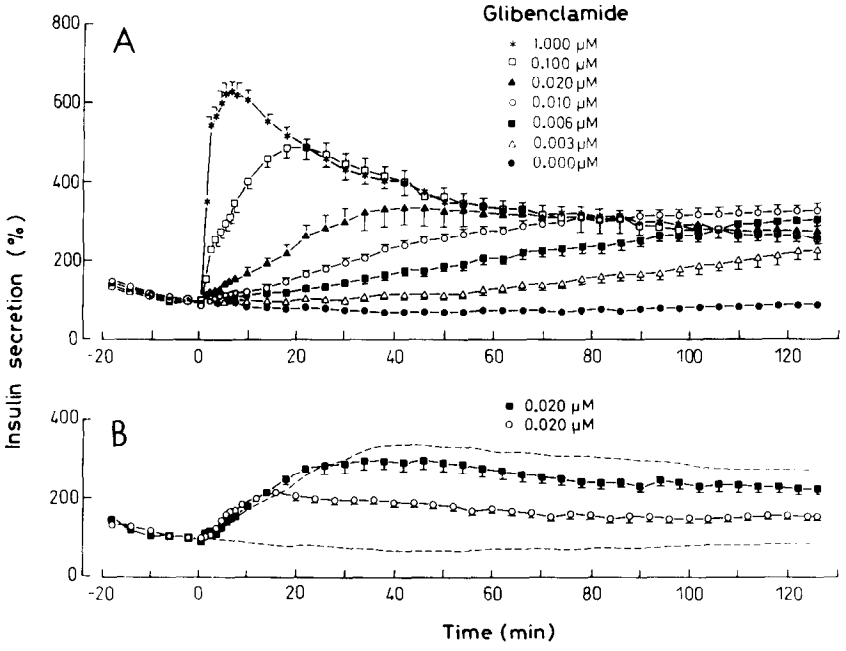


Fig. 4. Concentration-dependent effects of glibenclamide on the kinetic of insulin secretion by mouse pancreatic islets. All control and test media contained 10 mM D-glucose. Values in the curves are means ± SE of results from 6 separate experiments. (A) From zero time to min 128 the test media contained the indicated total glibenclamide concentrations. (B) From zero time to min 10 (open circles) or to min 20 (filled squares) the test media contained 20 nM glibenclamide (total concentration) and subsequently the perifusions were run in the sole presence of 10 mM D-glucose. To facilitate comparison, the traces symbolized by ● or ▲ in part A are shown as dashed lines.

*Reversal of sulfonylurea-induced block of ATP-dependent $K^+$ channels*

To evaluate the role of ATP-dependent $K^+$ channels in producing the above differences in reversibility we studied the kinetics of disappearance of $K^+$ channel block. Since the spontaneously occurring "run-down" of the ATP-dependent $K^+$ channels prevented a clear detection of very slow responses [3], recovery from block by sulfonylureas was accelerated by the addition of diazoxide. After removal of a maximally (100 nM) or submaximally (10 nM) effective glipizide concentration, whole-cell $K^+$ currents

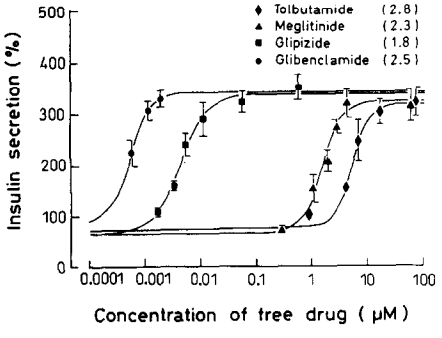


Fig. 5. Dependence of insulin secretion on the free concentrations (logarithmic scale) of tolbutamide, meglitinide, glipizide and glibenclamide. The secretory rates at min 58 (tolbutamide, meglitinide, glipizide) or min 126 (glibenclamide) were taken from the experiments described in Figs. 3 or 4A, respectively. In the case of glibenclamide only rates observed at total concentrations lower than 20 nM were used. The corresponding free drug concentrations were calculated using the relations illustrated in Fig. 2A and the concentration–effect relations were fitted as described in the Materials and Methods section. The corresponding Hill coefficients are indicated in parentheses. The values in the curves are means ± SE.

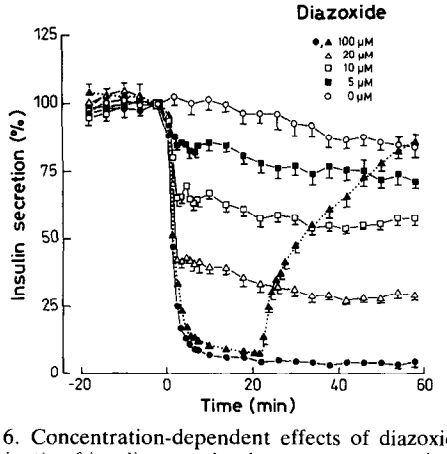


Fig. 6. Concentration-dependent effects of diazoxide on the kinetic of insulin secretion by mouse pancreatic islets. All control and test media contained 40 mM D-glucose. After perifusion for 60 min with control medium, the media contained the indicated total diazoxide concentrations from zero time to min 20 (▲) or min 60 (all other curves). Values in the curves are means ± SE of results from 6 separate experiments.

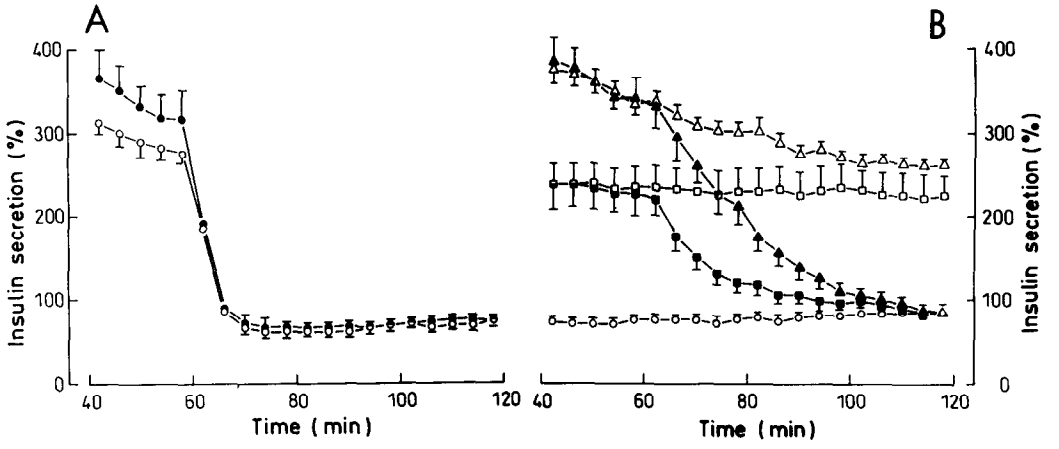


Fig. 7. Reversal of insulin release induced by tolbutamide, meglitinide and glipizide in mouse pancreatic islets. All control and test media contained 10 mM D-glucose. Values in the curves are means ± SE of results from 6 separate experiments. (A) The media contained tolbutamide (30 μM, ●; total concentration) or meglitinide (7 μM, ○; total concentration) from zero time to min 60. (B) The media contained glipizide (0.10 μM, ▲; 0.01 μM, ■; total concentrations) from zero time to min 60 or glipizide (0.10 μM, △; 0.01 μM, □; total concentrations) from zero time to min 120. ○, no drug was present from zero time to min 120.

increased up to the end of the test period (Fig. 9A and B). However, reversibility of submaximal effects of glibenclamide was not observed during this period (Fig. 9C). The slight increase in $K^+$ currents within the first min after start of medium change was probably caused by diazoxide-induced opening of $K^+$ channels previously blocked by ATP of the pipette solution.

*Membrane binding of hypo- and hyperglycaemic drugs*

Binding of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide to islet-cell membranes was determined for comparison with their effects in B-cells. Pilot tests at room temperature indicated that binding of [$^3$H]glibenclamide (0.3 or 1 nM) to islet-cell microsomes reached equilibrium within 15 min and was proportional to the microsomal protein concentration in the incubation volume up to 0.2 mg/ml (results not shown). Binding was saturable and non-specific binding was very low (Fig. 10A). In the 2 equilibrium binding experiments the Hill coefficient for specific binding was 0.93 or 1.16 and the Scatchard plots showed a single type of binding sites with a dissociation constant ($K_d$) of 0.40 or 0.46 nM and a maximal number of binding sites ($B_{max}$) of 0.93 or 1.38 pmol/mg protein (Fig. 10A, inset). Increasing concentrations of tolbutamide,

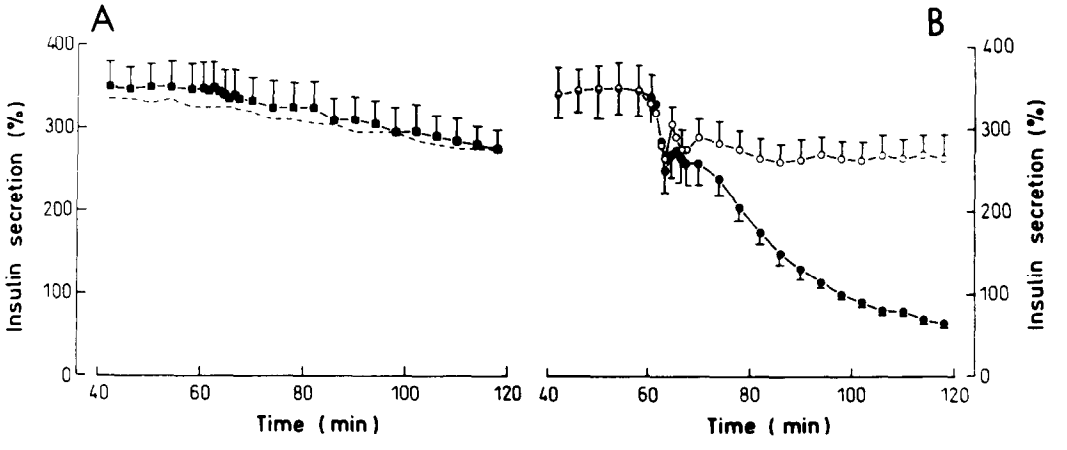


Fig. 8. Reversal of insulin release induced by glibenclamide in mouse pancreatic islets. All control and test media contained 10 mM D-glucose. Values in the curves are means ± SE of results from 6 separate experiments. (A) The media contained glibenclamide (20 nM, ■; total concentration) from zero time to min 60. To facilitate comparison, the trace symbolized by ▲ in part A of Fig. 4 is shown as a dashed line. (B) The media contained glibenclamide (20 nM, total concentration) from zero time to min 60 (●) or min 120 (○). From min 60 to min 120 the media contained diazoxide (100 μM; total concentration).

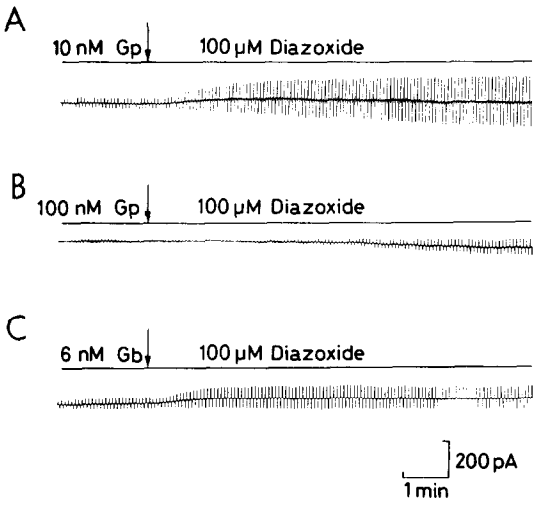


Fig. 9. Reversal of glipizide- or glibenclamide-induced block of ATP-dependent $K^+$ currents in mouse pancreatic B-cells. B-cells were incubated for 1–2 hr in the presence of the indicated total concentrations of glipizide (Gp) or glibenclamide (Gb). Recordings A–C started about 1 min after establishing the whole-cell configuration of the patch-clamp technique. At the times marked by arrows, exchange of sulfonylureas for diazoxide was started by perfusion of the incubation chamber. The traces shown are representative of 5 (A), 3 (B) or 9 (C) experiments which gave similar results. All bath solutions contained 2 mg/ml albumin.

meglitinide and glipizide inhibited the specific binding of [$^3$H]glibenclamide to islet-cell microsomes and half-maximally inhibitory concentrations ($IC_{50}$) were 22.2 μM, 5.7 μM or 14.1 nM, respectively (Fig. 10B). These values, the $K_d$ value of [$^3$H]glibenclamide (0.43 nM) and its free concentration were used to calculate the $K_d$ values in Table 1 as described in [30]. The Hill coefficients for the effects of tolbutamide, meglitinide or glipizide were −0.85, −0.82 or −0.96, respectively. Diazoxide displaced labelled glibenclamide only weakly ($IC_{50}$ value greater than 200 μM; Fig. 10B).

Figure 11 shows that after equilibration with 0.3 or 4 nM of [$^3$H]glibenclamide the time-courses of dissociation of label were biphasic. There was no complete reversibility of specific binding within 90 min. Analysis of the kinetic revealed two components of specific [$^3$H]glibenclamide binding which dissociated at half-lives of around 3 and 30 or 50 min.

## DISCUSSION

### *Potency of hypoglycaemic drugs*

Determination of the insulin-releasing potency of sulfonylureas should fulfil three criteria for correlation with their activity and affinity at the receptor level. (1) The free drug concentrations must be known for the experimental conditions under which insulin secretion is investigated. The real concentrations of free glibenclamide in our albumin-

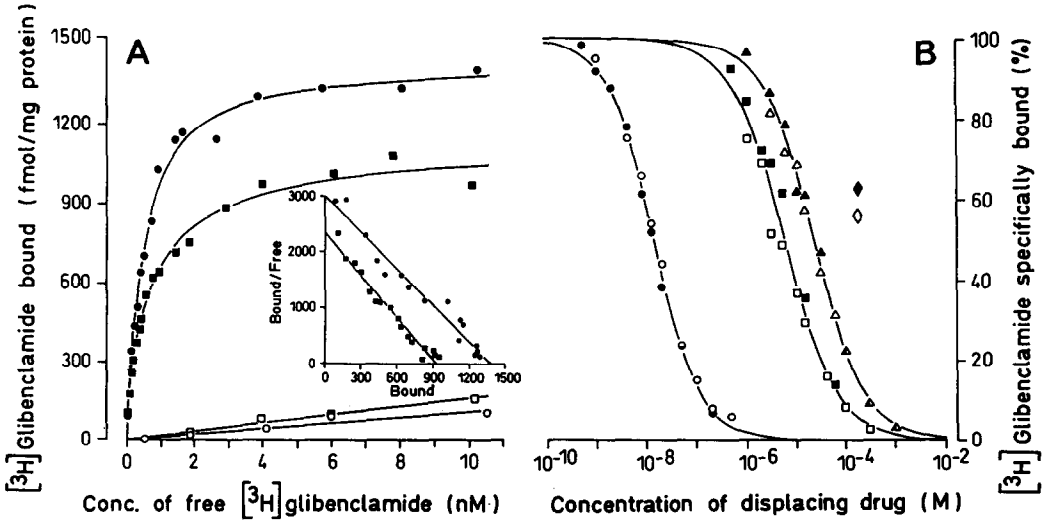


Fig. 10. Binding of [$^3$H]glibenclamide to microsomes from ob/ob mouse islets. (A) Equilibrium binding was performed at room temperature for 60 min in the absence (filled symbols) or presence (open symbols) of 1 μM unlabelled glibenclamide using 1 ml of buffer containing [$^3$H]glibenclamide (0.03–10.5 nM) and microsomal protein (40 μg/ml). Values in the curves were measured in 2 separate experiments using separate membrane preparations (circles or squares). Inset, Scatchard plots of the specific [$^3$H]glibenclamide binding in the 2 experiments. The lines were calculated by linear regression analysis ($r$ = 0.98 in both experiments). (B) Inhibition of [$^3$H]glibenclamide binding by tolbutamide (triangles), meglitinide (squares), glipizide (circles) or diazoxide (rhombs) was measured at room temperature in 1 ml of buffer containing [$^3$H]glibenclamide (0.2 nM), microsomal protein (40 μg/ml) and the indicated concentrations of displacing drug. Incubations lasted 60 min. Nonspecific binding was 3% of total binding. Results are presented as percentages of specific binding of [$^3$H]glibenclamide in the absence of other drugs. Values in the curves were measured in 2 separate experiments using separate membrane preparations (open or filled symbols).

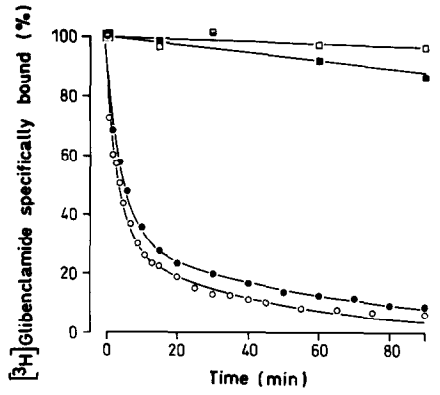


Fig. 11. Reversal of [$^3$H]glibenclamide binding to microsomes from ob/ob mouse islets. Microsomes (80 μg/ml of protein) were preincubated for 15 min with 0.3 nM (open symbols) or for 60 min with 4 nM (filled symbols) of [$^3$H]glibenclamide. At zero time dissociation was induced by addition of unlabelled glibenclamide (1 μM final concentration) to an aliquot (circles) and the remaining preincubation medium served as control (squares). Aliquots were filtered at the time intervals indicated. Nonspecific binding was determined using preincubations in the presence of 1 μM unlabelled glibenclamide and did not vary significantly up to min 90. Specific binding is given as percentage of specific binding at zero time. Values in the curves represent the mean of results from 2 separate experiments performed at room temperature. Considering the time courses of the control curves, the analysis of the dissociation curves gave the following dissociation rate constants: $k_1 = 0.023\ min^{-1}$ and $k_2 = 0.287\ min^{-1}$ (open symbols); $k_1 = 0.014\ min^{-1}$ and $k_2 = 0.214\ min^{-1}$ (filled symbols). At zero time the slow components amounted to 30% (open symbols) or 31% (filled symbols) of the total ligand specifically bound.

containing perifusion media were eight-fold higher than expected from calculations using binding parameters defined for human albumin in phosphate buffer [23]. This confirms that binding of sulfonylureas depends on the type of albumin and the ionic composition of the solution [31]. (2) Secretory rates in steady states of drug action should be used since the kinetics of insulin release in response to glipizide or glibenclamide were dependent on their concentrations (see Results). (3) Testing of sulfonylureas in resting B-cells should be avoided. Otherwise, varying concentrations of sulfonylureas are required to depolarize the B-cells to the threshold at which insulin release is initiated and the concentration–response curves are shifted to the right. Therefore, our perifusion media were supplemented with a glucose concentration (10 mM) slightly stimulatory in isolated mouse islets [21].

Recent studies on tumor cells derived from hamster B-cells (HIT cells) or rat B-cells (CRI-G1-cells) reported that 112 or 200 nM, respectively, of total glibenclamide were half-maximally effective on insulin release in the presence of 1 mg/ml of bovine albumin [5, 32]. According to Fig. 2 these concentrations corresponded to about 25 and 50 nM of free glibenclamide. These very high values were due to the experimental design chosen by the authors, i.e. short incubation periods of 10–15 min in the presence of substimulatory glucose concentrations. Figure 4A indicates that the steady state of action of low glibenclamide concentrations is not reached within 10–15 min. For the same reasons the $EC_{50}$ value for glipizide was also higher than in the present study [5]. However, in ATP-depleted insulinoma cells derived from rat B-cells (RIN cells) 0.06 or 0.5 nM of free glibenclamide or glipizide, respectively, were half maximally effective on $^{86}Rb$ efflux which reflects the activity of the ATP-dependent $K^+$ channels [10]. It is unclear why these values were 7–8 times lower than the $EC_{50}$ values for effects on normal B-cells (Table 1) though the tumor cells were exposed to sulfonylureas for only 20 min.

*Kinetics of secretory responses to hypo- and hyperglycaemic drugs*

Increase in insulin secretion was slowed down more and more with lowering of the concentrations of glipizide or glibenclamide. The time courses did probably not reflect drug association with a sulfonylurea receptor freely accessible from the extracellular space because binding of glibenclamide (0.3–1 nM) to islet microsomes reached equilibrium more rapidly

Table 1. Biological activities and binding affinities of hypo- and hyperglycaemic drugs

| Drug | Plasma* concentration | Islet† $EC_{50}$ | B-cell‡ $EC_{50}$ | Binding site§ $K_d$ |
|---|---|---|---|---|
| Tolbutamide | 6–25 μM (94) | 5 μM | 7 μM | 15 μM |
| Meglitinide | | 1.6 μM | 0.5 μM | 4 μM |
| Glipizide | 4–40 nM (98) | 4 nM | 4 nM | 10 nM |
| Glibenclamide | <10 nM (>99) | 0.5 nM | 0.4 nM | 0.4 nM |
| Diazoxide | 5–17 μM (92) | 11 μM | 16 μM | >140 μM |

Free drug concentrations are indicated.

* Calculated from the binding percentages given in parentheses (taken from Refs 33–36) and typical therapeutic plasma concentrations [36–39].

† Values are for effects on insulin release observed 126 min after addition of glibenclamide or 58 min after addition of the other drugs (see Results).

‡ Values are for effects on the ATP-regulated $K^+$ channel and were calculated from published data [2, 3] using the binding percentages shown in Fig. 2.

§ Values are for binding to microsomes from pancreatic islets and were calculated from results of equilibrium binding or competitive inhibition assays (see Results).

than the secretory responses to similar concentrations of free glibenclamide (see Results). Assuming penetration of sulfonylureas towards their receptor via the lipid phase of the B-cell plasma membrane, uptake into the cell interior could compete with receptor binding. It has been clearly shown that glibenclamide progressively accumulates in B-cells [4, 6]. On the other hand, tolbutamide and glipizide were proposed to be restricted to the outer half of the B-cell plasma membrane since the distribution volume for these drugs in isolated islets only slightly exceeded that for extracellular space markers [4]. However, at pH 7.4 media supplemented with sulfonylureas or meglitinide contain small proportions of their undissociated forms which are highly lipid-soluble (see Fig. 1) and therefore diffuse into the plasma membrane and then enter the intracellular water phase. The B-cell membrane potential (at rest around −60 to −70 mV, [40]) and the Nernst equation predict that the concentration of anionic form free in the B-cell cytosol is much lower than in the incubation medium [41]. This explains the outcome of the above studies on tolbutamide and glipizide uptake in islets. Glibenclamide is exceptional in that its apparent octanol/water partition coefficient at pH 7.4 exceeds the coefficients for tolbutamide, meglitinide and glipizide about 30-fold. Thus, accumulation of glibenclamide in intracellular membrane compartments is favoured and therefore retards the increase in the plasma membrane content of drug more than in the case of glipizide. However, when micromolar concentrations of sulfonylureas or meglitinide are applied, flux into intracellular pools seems to be insufficient to delay the increase in membrane content of drug.

At non-saturating concentrations of hypoglycaemic drugs, their rate of effectuation declined in the order tolbutamide = meglitinide > glipizide > glibenclamide. The same rank order was observed when comparing the rates of disappearance of insulin releasing effects. Reversal of glibenclamide-induced insulin release was so slow that it could be demonstrated only in the presence of a high diazoxide concentration (Fig. 8B). Diazoxide prevents blocking of $K^+$ channels by ATP [2, 3] and so allows insulin secretion to decrease immediately after diminishing the membrane content of sulfonylureas bound to their receptor. The slow reversibility of the secretory response to glibenclamide may be due to diffusion of drug from intracellular stores into the plasma membrane as exemplified by diazoxide which is also highly lipid-soluble at pH 7.4. Since diazoxide administration established new steady states of insulin secretion within a few minutes and micromolar drug concentrations were required, the dissociation of the receptor–drug complex seems to be too rapid to explain the protracted reversal of the inhibitory effects of diazoxide. However, in the case of glibenclamide the binding studies with islet-cell membranes suggest that the dissociation kinetic of the receptor–drug complex contributes to the very slow off-response after removal of glibenclamide. This view is supported by the observation that, after removal of glibenclamide, block of whole-cell $K^+$ currents did not disappear more rapidly than stimulation of insulin release (compare trace C in Fig. 9 with the trace with filled circles in Fig. 8B). In the whole-cell configuration of the patch-clamp technique small molecules (<1000 daltons) diffuse rapidly from the cell interior into the recording pipette [42].

*Membrane binding of hypo- and hyperglycaemic drugs*

In tumor cells the rank order of biological potency of hypoglycaemic drugs was the same as that of binding affinity suggesting that the identified binding sites were the receptors initiating the insulin-releasing effects of sulfonylureas [5, 10]. The same holds true for B-cells in pancreatic islets (Table 1). In islet-cell membranes the binding affinities of sulfonylureas and meglitinide were lower than in membranes of RIN insulinoma cells [9, 10] and higher than in membranes of the HIT-type B-cell line [5]. These differences may reflect special features of the tumor cell membranes. However, it seems possible that the results for HIT cell membranes were distorted by using high concentrations of membranes and tracer in the binding experiments. The $K_d$ values for binding of tolbutamide, meglitinide and glipizide to islet-cell membranes were about 3 times higher than the $EC_{50}$ values for their biological activities (Table 1). This probably indicates that more receptors are present than required for a maximal response (spare receptor concept, Ref. 43). The failure to demonstrate spare receptors in the case of glibenclamide may result from slight overestimation of the $EC_{50}$ values due to the very slow responses to low glibenclamide concentrations. The present experiments confirm that insulin-secreting cells contain a single type of binding site for sulfonylureas which lack cooperative site–site interaction [5, 10]. Therefore, the biphasic time-course of dissociation of glibenclamide binding (Fig. 11) indicates a complex reaction, e.g. isomerization of the receptor–drug complex [44]. Much higher concentrations of diazoxide were necessary for displacement of glibenclamide binding than for activation of ATP-regulated $K^+$ channels and inhibition of insulin release (Table 1). This finding supports the view that the effects of sulfonylureas or diazoxide result from interaction with different binding sites in the B-cell plasma membrane [3].

*Sulfonylurea receptors initiating hypoglycaemia*

The present study strongly suggests that blocking of the ATP-regulated $K^+$ channel in the B-cell is the sole mechanism by which sulfonylureas and meglitinide initiate insulin release. (1) A close correlation between $K^+$ channel-blocking and insulin-releasing potencies of the drugs exists (Table 1). (2) The rate of disappearance of $K^+$ channel block declines in the order tolbutamide = meglitinide > glipizide > glibenclamide (see Results and Ref. 3). The same rank order is seen when comparing the rates of disappearance of insulin-releasing effects. (3) Differences in the kinetics of responses to the drugs are due to special features of drug distribution. Allowing for these differences, the same maximum secretory rates are caused by saturating concentrations of sulfonylureas and meglitinide.

Opening of the ATP-dependent $K^+$ channel of the B-cell might be the major mechanism by which

Table 2. Lowest concentrations of free sulfonylurea effective on extrapancreatic cells.

| Drug | Hepatocyte | Myocyte | Adipocyte |
|---|---|---|---|
| Tolbutamide | 100 μM, [49] | 80 μM, [50] | |
| Tolazamide* | 250 μM, [51] | 80 μM, [52] | 2 μM, [53] |
| Gliquidone* | 3 μM, [54] | | |
| Glipizide | 5 μM, [55] | | |
| Glibenclamide | 70 nM, [56] | 100 nM, [50] | 40 nM, [57] |

The lowest concentrations reported to be effective *in vitro* were taken from the cited references. These data and the binding percentages shown in Fig. 2 were used to calculate the indicated free concentrations.

* Typical therapeutic plasma concentrations of tolazamide or gliquidone are 20–130 μM or 0.3–3 μM, respectively [58, 59] and about 10% or <1% of drug are free, respectively [58, 60].

diazoxide initiates hyperglycaemia [2]. This view is supported by the similarity of the diazoxide concentrations effective in pancreatic B-cells and in therapy (Table 1). The free concentrations of tolbutamide, glipizide and glibenclamide which are effective on single B-cells and isolated pancreatic islets are also in the range of therapeutic plasma concentrations of the free drugs (Table 1). In this range, direct effects of sulfonylureas on the glucagon-secreting A-cells have not been clearly demonstrated in pancreatic islets exposed to normal or high glucose concentrations [45–47]. This is not surprising since A-cells are devoid of ATP-regulated $K^+$ channels [48]. With the exception of an isolated observation in adipocytes, sulfonylurea concentrations required for induction of metabolic responses in extrapancreatic cells have been well beyond the range of therapeutic concentrations (Table 2). In adipocytes a low threshold level of tolazamide for enhancement of insulin-stimulated hexose uptake has been observed [53]. However, it is questionable whether this finding is relevant to *in vivo* conditions [61]. There is thus compelling evidence that the hypoglycaemic effect of sulfonylureas results solely from their interaction with the receptor identified in the B-cell plasma membrane.

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