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Evidence for more than One Binding Site for Sulfonylureas in Insulin-secreting Cells

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Abstract—Specific binding of both [^{3}H]glibenclamide and [^{3}H]gliquidone has been observed in a particulate fraction of insulin-secreting rat tumour (RIN m5F) cells. The binding of both the labels was time-dependent, of high affinity (including a low affinity binding site), saturable and reversible. The rank order of inhibition of [^{3}H]glibenclamide binding was glibenclamide > gliquidone > AG-EE 388 = AG-EE 86 = AG-EE 319 > AG-EE 436 (AG coded drugs are benzoic acid derivatives which lack the sulfonylurea moiety of sulfonylureas). The K_ds of high affinity binding for glibenclamide and gliquidone were 0·08 and 1·3 nM, respectively. When [^{3}H]gliquidone was used as the labelled compound this rank order of binding and the affinities of drugs were different, e.g. glibenclamide was less potent than gliquidone. The K_d values of high affinity binding to the [^{3}H]gliquidone binding site were 810 and 79 nM with respect to glibenclamide and gliquidone. The binding site labelled by [^{3}H]gliquidone, in contrast to that labelled by [^{3}H]glibenclamide, was not able to discriminate between the two enantiomers AG-EE 319 and AG-EE 436. The data indicate that there are different binding sites for glibenclamide and gliquidone in RIN m5F cells. In extension to data of other groups it is speculated that there exists more than one specific binding site for sulfonylureas and other related compounds, e.g. benzoic acid derivatives and that sulfonylureas behave differently not only in quantitative but in qualitative terms as well.

The molecular mechanism of the insulin-releasing capability of sulfonylureas includes their binding to a plasma membrane site of the B-cell (Hellman & Täljedal 1975; Hellman et al 1971, 1973; Sehlin 1973; Täljedal 1974). The sulfonylurea receptor itself is thought to be the ATP-modulated K^+ channel or a structure closely related to that channel which controls its activity (Schmid-Antomarchi et al 1987; Zünkler et al 1988). So far it is not known whether all members of the sulfonylurea family act in a similar way, and whether, in addition to well-known quantitative differences, there exist qualitative differences between various sulfonylureas. As far as binding to islet cell membranes is concerned, a binding affinity of 40 nM for glibenclamide was described (Joost et al 1980). Insulin secreting cell lines such as HIT cells (hamster insulinoma) have been used to identify a binding site for tolbutamide (Gaines et al 1987) the affinity of which (IC50 = 6 μM) is close to the in-vitro half-maximal insulin stimulatory concentration (EC50 = 3 μM) (Joost & Hasselblatt 1979). In addition, a glibenclamide binding site in the HIT cell fraction has been identified, the affinity of which (K_d = 2·0 nM) is close to the in-vitro half-maximal insulin stimulatory concentration (EC50 = 5·5 nM) (Gaines et al 1988). Employing a particulate fraction of an insulin secreting rat tumour cell line (RIN m5F) it was the aim of the present study to investigate the binding characteristics of various sulfonylureas by using two radioactive labelled sulfonylureas and to evaluate the possibility of more than one binding site, a problem that has not yet been addressed.

Some benzoic acid derivatives that are structurally related to the non-sulfonylurea moiety of sulfonylureas were also included in this study since they show effects (Garrino & Henquin 1988; Garrino et al 1985, 1986; Henquin et al 1987) that are similar to those of sulfonylureas (Henquin 1980) i.e. inhibition of ^{86}Rb efflux, depolarization of the B-cell membrane, induction of electrical acitivity, stimulation of ^{45}Ca efflux and potentiation of insulin release. Another reason to include these drugs is that enantiomers are available.

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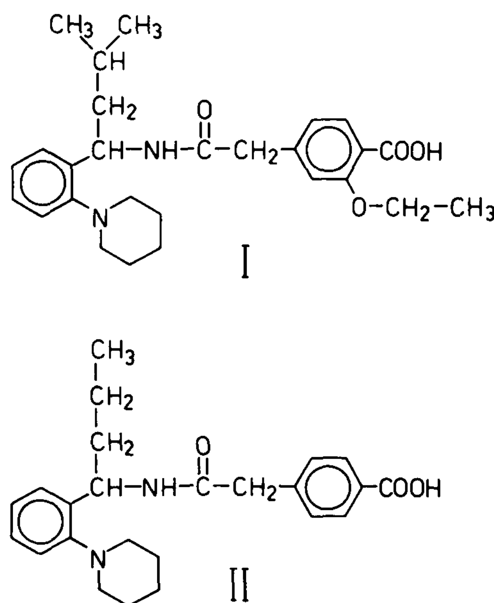

Materials and Methods

Chemicals

Ham's F12, fetal calf serum, penicillin, streptomycin were from Seromed, Berlin (West Germany). The following were purchased: HEPES (2-(4-(hydroxyethyl)-1-piperazinyl)-eth-

anesulfonic acid) from Sigma Chemical Co. (St. Louis, Missouri); bovine serum albumin (BSA) fraction V from Miles Laboratories (Elkhart, Indiana); [^{3}H]glibenclamide (2·3 TBq g^{-1} spec. act.; purity 98% by TLC) was purchased from Hoechst AG (Frankfurt/Main, West Germany), [^{3}H]gliquidone (191·6 GBq g^{-1} spec. act.; purity 96% by TLC) was kindly provided by Dr Karl Thomae GmbH, Biberach an der Riss, West Germany. The benzoic acid derivatives AG-EE 388 (I), AG-EE 86 (II) and the two enantiomers of AG-EE 86, i.e. AG-EE 319 [(+)-enantiomer] and AG-EE 436 [(−)-enantiomer], were synthesized by Dr W. Grell (Dr Karl Thomae GmbH, Biberach an der Riss, West Germany). The enantiomeric purity was as follows: (+)-enantiomer, ee = 95·8 ± 0·4, (−)-enantiomer, ee = 99·7 ± 0·2.

Cell culture

RIN m5F cells generously provided by Dr C. Wollheim, Geneva, Switzerland, were grown in plastic culture bottles for 4–6 days (half confluent: 1–2 × 10^6 cells mL^{-1}) in RPMI medium supplemented with 10% (v/v) foetal calf serum, 100 units of penicillin mL^{-1} and 0·1 mg of streptomycin mL^{-1}. On the day of the experiment (passage No. 80–94) cells were detached with 0·025% trypsin plus 0·1% EDTA in a Ca^{2+}, Mg^{2+}-free buffer, and isolated cells were allowed to recover in a 3 h spinner culture (same medium). Then cells were washed and resuspended in Krebs-Ringer buffer containing 10 mM HEPES and 0·5% bovine serum albumin. Subsequent experiments were performed with crude membrane fractions but not with whole cells since e.g. glibenclamide is trapped (internalized) by cells (Carpentier et al 1986) and the cell-associated glibenclamide may obscure binding to superficial receptors. Previous data obtained from intact islets of obese-hyperglycaemic mice, indicating that [^{3}H]glibenclamide binding is not affected by tolbutamide (Hellman 1974) and [^{3}H]tolbutamide binding is inhibited by glibenclamide (Sehlin 1973), are therefore hard to interpret.

Preparation of a particulate fraction

RIN m5F cells were washed with 3 mL of Krebs-Ringer buffer with HEPES and with 0·5% albumin, scraped off with a "rubber policeman" and centrifuged. The pellet was suspended in 5 mL KRH buffer (without albumin) and sonicated (Econoclean, Spirig, Rappersvil, Switzerland) (35 Hz, 150 W) at 4°C. A pellet was obtained at 30 000 *g* (20 min) which was resuspended in roughly 7 mL of the same buffer to give a final protein content of about 150 μg/200 μL sample.

Binding experiments

100–200 μg cell protein was incubated in 200 μL KRH-buffer pH 7·4 at 22°C; then 0·32 nM [^{3}H]glibenclamide or 10 nM [^{3}H]gliquidone with or without 10 μM unlabelled glibenclamide, or gliquidone, was then added to give a final volume of 300 μL. The incubation was terminated by cooling the samples to 4°C, which were then filtered through glass fibre filters (Whatman GF/C, Whatman Ltd, Springfield Mill, Maidstone, UK) after having been rinsed shortly before. The filters were washed twice with 1 mL KRH-buffer. All steps were carried out at 4°C. Filters were immersed in a scintillation cocktail (Rothiszint Mini, Roth GmbH, Karlsruhe, West Germany), shaken vigorously and counted in a β-scintillation counter (Packard Isocap/300 Nuclear-Chicago, Des Plaines, IL). Results of the samples were corrected for protein (see below). Each value was obtained as duplicates from at least four independent experiments. Binding of both the labelled sulfonylureas was proportional to membrane protein concentrations between 40 and 240 μg/200 μL (data not shown).

All experiments were performed in the absence of albumin because control experiments in the presence of 0·5% albumin revealed a 100 times lower binding affinity of glibenclamide which correlates well with its high plasma protein binding of >99% (Crooks & Brown 1974). Moreover, the protein binding of sulfonylureas may be different among themselves and from that of the benzoic acid derivatives used, a factor that might obscure the results. All binding data, therefore, can only be compared with insulin secretion data corrected for the amount of drug bound to albumin.

Protein determination

The protein content of the solubilized particulate fractions (solubilized with 0·1 M NaOH) was measured using bovine serum albumin as a standard (Bradford 1976).

Scatchard analysis

To establish the apparent receptor affinity (dissociation constant, K_d) competitive inhibition of a specific ^{3}H-labelled compound by varying concentrations of its unlabelled analogue was analysed by fitting plots of bound hormone versus free hormone with a non-linear least-squares computer program that analysed the data in terms of one non-saturable and one or two saturable components (Dixon 1974).

In-vivo testing

To determine the blood sugar lowering efficacy of the test compounds in-vivo, the test procedure chosen was: groups of 5 female rats (200–220 g) were fasted overnight. Compounds (tested at at least 4 different dosages) were suspended in tylose and given by gavage. Just before, and 60, 120 and 180 min after dosing blood was taken retro-orbitally. Blood sugar was determined automatically with Glucoquant Boehringer Mannheim, West Germany (hexokinase/glucose-6-phosphate dehydrogenase method).

Results

Time course of binding

The binding of [^{3}H]glibenclamide and [^{3}H]gliquidone to a particulate fraction of RIN m5F cells at 22°C was rapid with one-half maximal binding occurring within 15 min and maximal binding at 30 to 40 min (Fig. 1). Maximal binding was 2·48 and 4·81% of total radioactivity per mg protein for [^{3}H]glibenclamide and [^{3}H]gliquidone, repectively. Non-specific binding (determined in the presence of 10 μM unlabelled glibenclamide and gliquidone, respectively) was 0·64 and 2·74% of added radioactivity per mg protein; it was high with respect to gliquidone but not due to impurity (see Materials and Methods). All subsequent binding studies were carried out for 45 min. The specific binding was reversible: irreversible binding (dissociation experiment) was

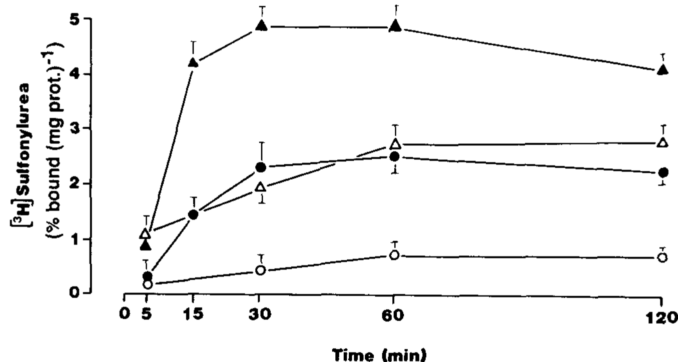

FIG. 1. Time course of [^{3}H]glibenclamide and [^{3}H]gliquidone binding to a particulate fraction of RIN m5F cells. 100–200 μg cell protein was incubated in 300 μL buffer for 0 to 120 min at 22°C with 0·32 nM [^{3}H]glibenclamide (●) or 10 nM [^{3}H]gliquidone (▲) in the absence or presence of either 10 μM unlabelled glibenclamide (○) or gliquidone (△). Results are expressed as % bound per mg protein. Each value represents the mean ± s.e.m. of 5 experiments.

less than 4% in three experiments for both the labelled ligands.

[^{3}H]Glibenclamide experiments

The ability of several sulfonylureas and benzoic acid derivatives to inhibit total [^{3}H]glibenclamide binding to a particulate fraction of RIN m5F cells was examined. Inhibition of specific [^{3}H]glibenclamide binding by unlabelled glibenclamide was sigmoidal, and not complete over seven orders of magnitude (Fig. 2). Binding analysis of a saturation experiment (competition-inhibition curve) generated with unlabelled glibenclamide revealed two binding sites (curvilinearity) with K_d values of 0·08 ± 0·028 nM and 187 ± 36 nM (mean + s.e.m., n = 6), which corroborates results obtained by Geisen et al (1985) for the relevant high affinity site using a transplantable insulin releasing tumour. In contrast, gliquidone, AG-EE 388, AG-EE 86, and its enantiomer AG-EE 319 were 10 or more times weaker, whereas its other enantiomer AG-EE 436 showed only minimal inhibition, i.e. it was 6000 times less potent (Table 1) compared with glibenclamide.

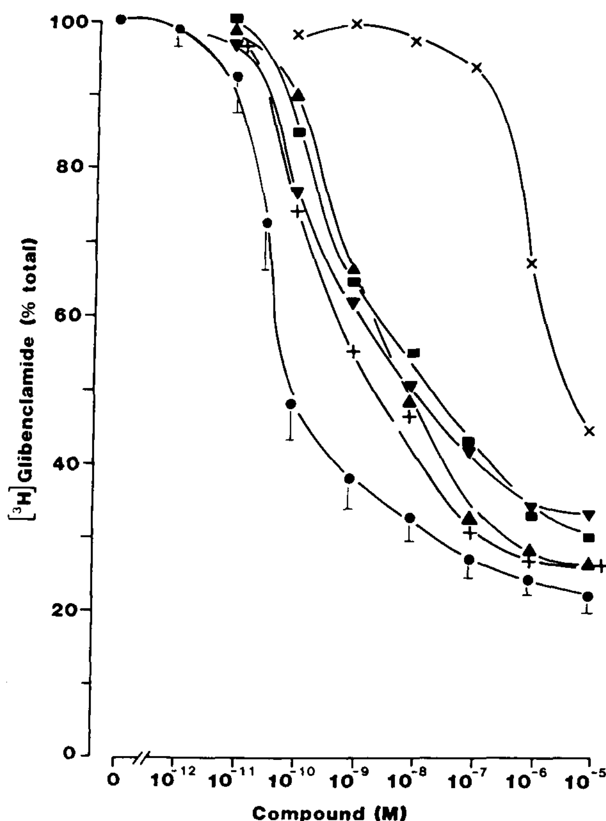

FIG. 2. Inhibition of total [^{3}H]glibenclamide binding to a particulate fraction of RIN m5F cells. 100–200 μg cell protein was incubated in 300 μL buffer for 45 min at 22°C with 0·32 nM [^{3}H]glibenclamide and increasing concentrations of drugs: glibenclamide (●), gliquidone (▲), AG-EE 388 (+), AG-EE 86 (■), AG-EE 436 (×), AG-EE 319 (▼). Each value represents the mean ± s.e.m. of 4 experiments.

[^{3}H]Gliquidone experiments

The ability of several sulfonylureas and benzoic acid derivatives to inhibit total [^{3}H]gliquidone binding to a particulate fraction of RIN m5F cells was also examined. Inhibition of specific [^{3}H]gliquidone binding by unlabelled gliquidone was sigmoidal, and was not complete over four orders of magnitude (Fig. 3). Again, a relatively high non-specific binding of approximately 50% as in Fig. 1 was found. This feature appears typically when gliquidone is used as a load (Kaubisch et al 1982). Binding analysis of the competition-inhibition curve generated with unlabelled gliquidone revealed two binding sites with K_d values of 79 ± 24 nM and 31 ± 12 μM (mean ± s.e.m., n = 4). In contrast, glibenclamide was 10 times less potent in inhibiting the binding of gliquidone, and AG-EE 388, AG-EE 86 and both its enantiomers AG-EE 319 and AG-EE 436 were about 600 times weaker, whereas AG-EE 86 was equipotent.

In-vivo experiments

As is evident from the data shown in Table 1 the sulfonylurea compounds glibenclamide and gliquidone exhibit in that model comparable ED10 values at all time points measured. The benzoic acid derivatives AG-EE 86 and AG-EE 388 are much more potent (about 15 times in case of AG-EE 388 compared with the above mentioned compounds). Whereas the (+)-enantiomer of AG-EE 86 (AG-EE 319) shows about twice the efficacy of the racemic mixture, the (−)-enantiomer (AG-EE 436) is a much weaker compound.

Table 1. Effect of sulfonylureas and benzoic acid derivatives on receptor binding and insulin release. Data of therapeutic plasma levels are from Joost & Hasselblatt (1979). Receptor binding was calculated from the concentration required for one-half maximal inhibition of ^{3}H-labelled drug binding. ED10 was the dose (mg kg^{-1}) to lower blood sugar in rats by 10 per cent.

Drug	Receptor binding [^{3}H]Glibenclamide K_{dI} (nM)	Receptor binding [^{3}H]Gliquidone K_{dI} (nM)	Therapeutic Plasma levels (nM)*	Therapeutic doses in man (mg)	Blood sugar lowering effect (rat) after 1 h	2 h ED10	3 h
Glibenclamide	0·08	810	4	2·5–15	0·122	0·138	0·098
Gliquidone	1·3	79	10	15–90	0·128	0·113	0·106
AG-EE 388	1·08	5200	n.d.	n.d.	0·007	0·005	0·005
AG-EE 86	1·04	73	n.d.	n.d.	0·016	0·027	0·040
AG-EE 319	0·96	4400	n.d.	n.d.	0·008	0·022	0·039
AG-EE 436	535	> 5000	n.d.	n.d.	0·43	2·49	11·19

*Corrected for protein binding.
n.d. = not determined.
K_{dI} = high affinity binding.

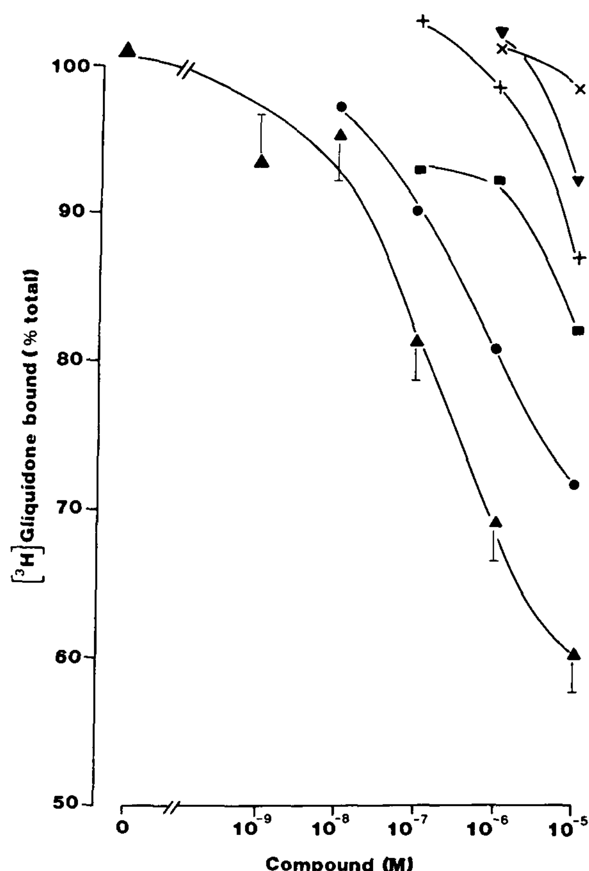

FIG. 3. Inhibition of total [^{3}H]gliquidone binding to a particulate fraction of RIN m5F cells. 100–200 μg cell protein was incubated in 300 μL buffer for 45 min at 22°C with 10 nM [^{3}H]gliquidone and increasing concentrations of drugs: glibenclamide (●), gliquidone (▲), AG-EE 388 (+), AG-EE 86 (■), AG-EE 436 (×), AG-EE 319 (▼). Each value represents the mean ± s.e.m. of 4 experiments. Note that the scale of y-axis is different from that in Fig. 2.

Discussion

[^{3}H]Glibenclamide binding site

Our data indicate that in a particulate fraction of RIN m5F cells binding sites for glibenclamide are present. The data are consistent with those of others (Schmid-Antomarchi et al 1987; Hellman et al 1976). In extension of the previous studies, criteria of binding such as a rank order of affinity of different drugs and stereospecificity with respect to the enantiomers AG-EE 319 and AG-EE 436, have been determined. The observed stereoselectivity (AG-EE 319 over AG-EE 436) of the receptors is indicative of the fact that the binding determined is not a simple accumulation of the drugs in the membrane or interaction with phospholipids, factors not excluded in earlier studies (Hellman et al 1976), but involves a specific interaction with a reactive site in the molecule. Our data corroborate those of others (Schmid-Antomarchi et al 1987) who show similar K_d values for glibenclamide (0·3 nM) and gliquidone (1 nM); however, we have demonstrated an additional binding site of low affinity which was substantiated by using an additional labelled sulfonylurea.

[^{3}H]Gliquidone binding site

From our data there are several lines of evidence that additional binding sites for sulfonylureas of the gliquidone type are present. First, we have shown for the first time that gliquidone possesses a higher affinity than other compounds when [^{3}H]gliquidone is used instead of [^{3}H]glibenclamide. Second, the rank order of binding of sulfonylureas and benzoic acid derivatives is different when [^{3}H]gliquidone is used instead of [^{3}H]glibenclamide. Third, a discrimination between enantiomers such as AG-EE 319 and AG-EE 436 is no longer as clear cut when [^{3}H]gliquidone, instead of [^{3}H]glibenclamide, is used. This different behaviour of the two binding sites labelled with either compound is obvious though there might exist cross-reactivity between both ligands and for each other's binding site. Recent photoaffinity labelling studies provide evidence for two sulfonylurea binding sites (140 kDa and 33 kDa); one of them possesses a low sensitivity towards cold glibenclamide (Kramer et al 1988) which corroborates our data well. The fact that in some investigations using islet microsomes or membranes only a single type of binding site was found (Gaines et al 1988; Panten et al 1989), may be due to different special features of the particulate fraction of RIN m5F cells which we used and the B cells used by those authors. However, even in that case

the fact that there exists a biphasic dissociation velocity (Panten et al 1989) should have resulted in site–site interactions which need further evaluation. The interpretation of sulfonylurea binding data may be even more complex since sulfonylureas have been shown to crossreact with α- and β-receptors of pancreatic islets (Cherskey & Altszuler 1984) depending on the sulfonylurea used.

The observed affinity values for gliquidone ($K_d = 79$ nM) are not in line with those of others (Kaubisch et al 1982) who found a K_{dl} of 6×10^{-11} mol L^{-1} for unlabelled gliquidone when labelled gliquidone was used, which is similar to the K_d of glibenclamide (5×10^{-11} mol L^{-1}). Thus, from the data of Kaubisch it appears that both ligands bind to the same sulfonylurea binding site and that there is only one binding site present. However, it is questionable whether the binding data of Kaubisch et al are of relevance as far as insulin secretion is concerned since these affinity data are far beyond the therapeutic gliquidone plasma concentration of 1 μM (= 10 nM non-protein-bound). Our data are also not consistent with other data of Kaubisch et al (1982) and those of Lupo & Bataille (1987) derived from cortical membranes, since a lower affinity for gliquidone than for glibenclamide was found when [³H]gliquidone was used as a tracer. However, their rank order of affinity does not fit the rank order of hypoglycaemic potency thereby shedding doubt on this method. Since depending on the labelled sulfonylurea (glibenclamide or gliquidone) a different affinity for gliclazide was found when a non-receptor containing preparation such as liposomes was used (Deleers & Malaisse 1984), it may be argued that membrane phospholipids, in part, obscure the results.

Correlation with in-vivo data

The maximum therapeutic plasma glibenclamide concentration is about 400 nM and it may be estimated that only about 4 nM is non-protein bound, i.e. biologically active (Table 1). When the perfused pancreas is used the EC50 for the insulin releasing capacity of glibenclamide is 0·02 mg mL^{-1} (= 50 nM) (i.e. 0·5 nM non-protein bound) and 0·5 nM (Joost et al 1980; Schwanstecher et al 1988). In our binding studies (absence of albumin) the K_{dl} of glibenclamide was 0·08 nM. This is lower than the therapeutic range (Table 1). However, Schwanstecher et al (1988) did not find a second binding site in pancreatic islet membranes which might also have obscured the relevance of their EC50 data. The same holds for gliquidone. The maximum therapeutic plasma gliquidone concentration is about 0·5 mg L^{-1} (= 1 μM; presence of plasma protein) or 10 nM (corrected for protein binding); therefore the K_{dl} of gliquidone (79 nM) is higher than the therapeutic range. The benzoic acid derivatives, except AG-EE 436, appear to be more potent in the in-vivo test than both the sulfonylureas tested (Table 1); this rank order does not correspond to either binding site labelled with [³H]glibenclamide or [³H]gliquidone.

Glibenclamide-induced insulin release has been suggested to be resolved into a "high-affinity" and a "low affinity" component (Norlund & Sehlin 1984). It was also suggested, but not proved, that the non-sulfonlyurea part of the sulfonylurea molecule (i.e. benzoic acid derivative) might interact with the "low affinity" component. Our data show that benzoic acid derivative binding is close to the range of low affinity binding of glibenclamide or gliquidone, rather than to its high affinity binding.

In conclusion, our data are indicative of more than one binding site of sulfonylureas in RIN m5F cells; this will have some impact on the possibility of quantitatively and qualitatively differing effects of various sulfonylureas.

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