Br. J. Pharmacol. (1990), **100**, 375–378

Glibenclamide is a competitive antagonist of the thromboxane A_2 receptor in dog coronary artery *in vitro*

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1 Glibenclamide, a sulphonylurea oral hypoglycaemic agent is a widely used antagonist of cromakalim-activated K^+ channels in smooth muscle.

2 In isolated ring segments of the large circumflex coronary artery from the dog, glibenclamide (1–30 μM) caused a concentration-dependent reduction in both spontaneous isometric force and contractions induced by U46619, a thromboxane A_2-mimetic.

3 Glibenclamide behaved as a competitive antagonist of U46619 with an estimated pK_B ($-\log K_B$) value of 6.2 by Schild regression analysis (slope 1.07).

4 Glibenclamide (30 μM) was apparently selective since it had no effect on the concentration-contraction curves to endothelin-1, noradrenaline or KCl.

5 We suggest that this additional property of glibenclamide should be considered in any smooth muscle study where active force is raised by either the exogenous application or endogenous generation of thromboxane A_2.

Introduction

The oral hypoglycaemic drug, glibenclamide, stimulates release of insulin from pancreatic acinar cells, probably by blocking an ATP-sensitive K^+ channel located in the plasma membrane (Schmid-Antomarchi *et al.*, 1987). It is also one of the most potent inhibitors of the cromakalim-activated K^+ channel in smooth muscle (Quast & Cook, 1988; Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze 1989a,b; Winquist *et al.*, 1989). Here we show that glibenclamide is also a competitive antagonist of the stable thromboxane A_2-mimetic, U46619, in dog isolated coronary artery. U46619 is a constrictor agent commonly used to induce active force in isolated blood vessels when studying vasorelaxation responses. Our results show that this competitive interaction of glibenclamide with U46619 is not shared with other constrictors such as endothelin-1, noradrenaline and KCl. This additional property of glibenclamide at the thromboxane A_2 receptor should be considered in any studies relating to smooth muscle reactivity where exogenous or endogenous thromboxane A_2-like compounds are present.

Methods

Coronary artery assay

Greyhound dogs (20–30 kg) of either sex were killed by an overdose of sodium pentobarbitone (80 mg kg^{-1}, i.v.). The heart was then removed and the circumflex coronary artery dissected from adhering connective tissue and fat. Ring segments of artery 3 mm long were mounted on stainless steel wires in 25 ml jacketed organ baths containing a modified Krebs solution of the following composition (mM): Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11, aerated with a gas mixture of 95% O_2 and 5% CO_2 and then allowed to equilibrate at 37°C for 30 min. Endothelium was removed from some rings by abrading the luminal surface with a tapered filter paper (Whatman No. 1, see Cocks & Angus, 1983). To examine direct relaxation effects of glibenclamide, some rings were stretched initially to 4 g and adjusted if necessary to 4 g, 30 min later (see Cocks & Angus, 1983). These rings were then allowed to equilibrate for 30 min before an EC_{80} concentration of U46619 (30 nM) or vehicle was added. Glibenclamide (3–30 μM) was then added when the spontaneous or U46619-induced contractions reached a plateau. For construction of cumulative concentration-contraction curves, all rings were stretched to an optimised resting circumference which corresponded to an equivalent transmural pressure of 100 mmHg. This normalisation procedure for large arteries has been published previously (see Angus *et al.*, 1986). After a further 60 min equilibration period, either glibenclamide or its vehicle (methanol) was added. A further 40 min was then allowed before cumulative concentration-contraction curves to U46619, noradrenaline, endothelin-1 or KCl were obtained. The noradrenaline curves were obtained in the presence of propranolol (1 μM) in endothelium-denuded rings. Only one concentration-contraction curve was obtained in any one ring.

Statistical methods

All contraction responses were normalised as percentages of the maximal contraction (F_{max}) to 75 mM KCl, which was added in the presence of the final concentration of each agonist. Each normalised contraction curve was then computer fitted with a logistic equation which gave estimates of the concentrations of the agent necessary to give EC_{10-90} of its maximum response. Full details have been published elsewhere (see Nakashima *et al.*, 1982; Angus *et al.*, 1986). The fitted EC_{50} value (expressed as the negative logarithm of the molar concentration) from each curve to U46619 was used to calculate the concentration ratio (CR) at the different concentrations of glibenclamide on any one day. Linear regression of the log (CR − 1) against glibenclamide concentration (log B), the 95% confidence limits for the line, the slope (b) and the x intercept (pK_B) were then determined (Snedecor & Cochran, 1967) from this Schild analysis (Arunlakshana & Schild, 1959). Unpaired *t* tests were used to test for statistical significance for data between rings and significance was accepted at the $P < 0.05$ level.

Preliminary experiments showed that equilibration of the arteries with glibenclamide before construction of the cumulative concentration-response curve to U46619 often caused a fall in resting force. This may have been due to antagonism of endogenous thromboxane A_2. If this was indeed correct, the control (zero glibenclamide) U46619 curve would be lower in sensitivity and the estimates of dose-ratios for the Schild analysis would also be low. A similar phenomenon has been discussed and analysed in detail regarding basal endogenous

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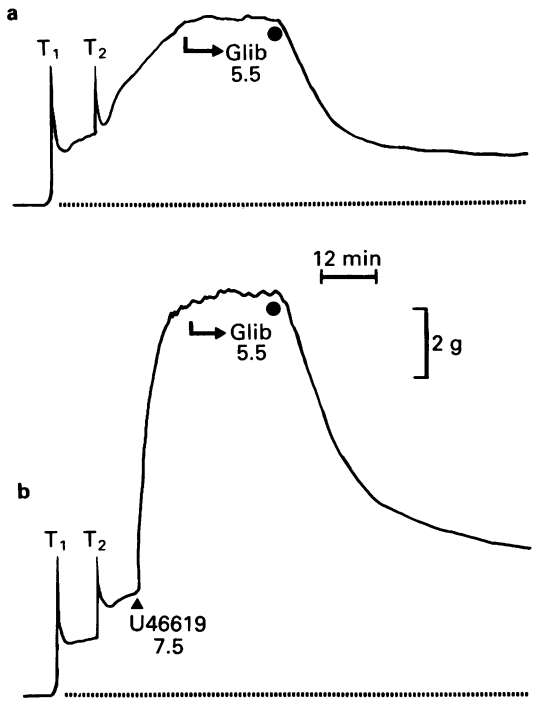

Figure 1 Original chart recordings showing the effect of glibenclamide (Glib) on spontaneously developed force (a) and U46619-induced force (b) in the greyhound circumflex coronary artery. Drug concentrations are given as $-\log$ M. T_1 and T_2 represent stretches of the tissues to 4 g force. Before the arrow the time calibration bar represents 40 min.

histamine release and acid secretion in the mouse stomach (Angus *et al.*, 1980). We therefore used a second analytical technique that does not rely on dose-ratios to estimate the pK_B value. These techniques and their advantages have been published elsewhere (Stone & Angus, 1978; Angus *et al.*, 1980). The entire data set of 313 data points was fitted with a reciprocal logistic model utilising the weighting facility of GLIM3 (General Linear Interactive Modelling; Stone & Angus, 1978). Concentration-response lines were fitted with a common slope and individual slopes to test for parallelism before applying the interactive, non-linear least-square curve

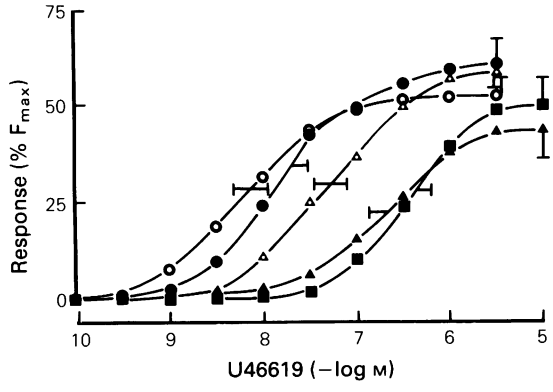

Figure 2 Cumulative concentration-contraction curves for U46619 in the greyhound circumflex coronary artery in the absence (○) and presence of 1 (●), 3 (△), 10 (▲) and 30 (■) μM glibenclamide. Values represent means of 6–7 experiments expressed as the percentages of F_{max} for each tissue. The vertical bars are 1 s.e.mean of the maximal response. The horizontal bars represent 1 s.e.mean of the EC_{50} ($-\log$ M) values at the point of intersection of each curve as determined from logistic curve fitting (see Methods). The EC_{50} values for U46619 were, respectively, 8.13 ± 0.18 ($n = 7$), 7.71 ± 0.18 ($n = 7$), 7.28 ± 0.18 ($n = 6$), 6.70 ± 0.17 ($n = 6$) and 6.35 ± 0.15 ($n = 6$) for 0, 1, 3, 10 and 30 μM glibenclamide. The maximum responses to U46619 (% F_{max}) for each concentration of glibenclamide were not significantly different from the control value, which was 51.8 ± 5.8%.

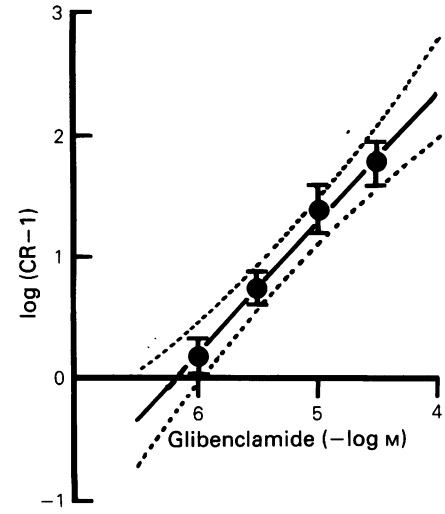

Figure 3 Graphical display of the antagonism of U46619 by glibenclamide according to Schild regression analysis. The solid line is the regression of the individual values from 6–7 experiments at each concentration of glibenclamide. The dotted lines represent the 95% confidence limits of the linear regression. The symbols represent the mean with 1 s.e.mean (vertical bars) values at each concentration of glibenclamide. Ordinate scale: concentration-ratio -1 (log scale). Slope = 1.07; $pK_B = 6.18$.

fitting approach of Waud (1975) to estimate the antagonist dissociation constant (K_B) and the parameter, n, equivalent to the slope in the Schild plot as a test of the competitive model. Finally, we used the 'Clark' plot (Stone & Angus, 1978; Stone, 1980) as a graphical display of how well the rightward displacement of the concentration-response curves conformed to the model of simple competitive antagonism.

Drugs

Drugs used and their sources were: U46619 (1,5,5-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid, Upjohn: Kalamazoo, U.S.A.), endothelin-1 (Auspep: Melbourne, Australia), (−)-noradrenaline bitartrate, indomethacin, 5-hydroxytryptamine creatinine sulphate, acetylcholine bromide, (±)-propranolol hydrochloride (all from Sigma, U.S.A.); glibenclamide (Daonil) and tolbutamide (Hoechst: Australia). All drugs were freshly prepared, and except for indomethacin and

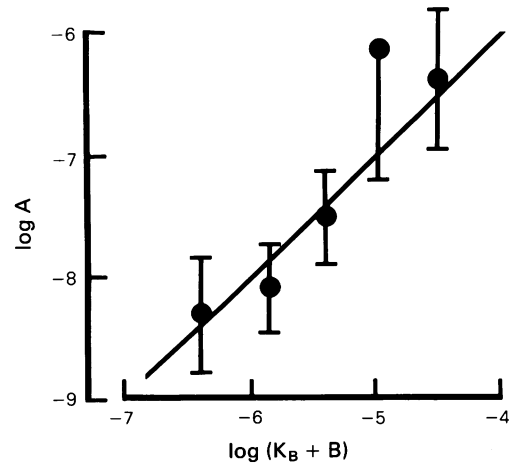

Figure 4 Graphical display ('Clark' plot) of the relationship between the level of agonist (A) (U46619, log M) and the level of antagonist (B + K_B) (glibenclamide, log M). Points shown display the degree of displacement of the mean concentration-response curves from the model of simple competitivity indicated by the solid line. The error bars are the 95% confidence limits. Note that the K_B was estimated by an interactive, non-linear least squares fitting approach using the entire data set (see Methods).

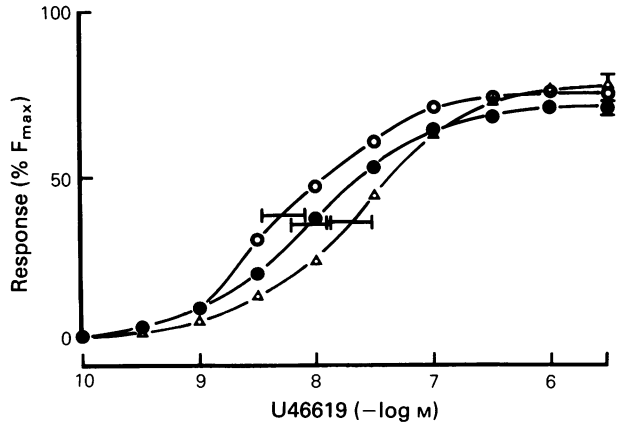

Figure 5 Concentration-contraction curves for U46619 in the absence (○) and presence of 3 (●) and 30 (△) μM tolbutamide. Data are expressed as described in Figure 2. The control EC_{50} (−log M) and maximum response (% F_{max}) were, respectively, 8.27 ± 0.18 and 74.5 ± 3.3 (n = 5). The EC_{50} and maximum response values for 3 and 30 μM tolbutamide were not significantly different from the control values.

glibenclamide, were dissolved in distilled water. Indomethacin was made up as a 0.1 M stock in 1 M Na_2CO_3 and then diluted in distilled water. Glibenclamide and tolbutamide were dissolved in 100% methanol to a final concentration of 0.01 M.

Results

Endothelium-intact rings developed spontaneous, active force ('tone', range: 1–7 g) within the 60 min equilibration period after their internal circumference had been set. By contrast, rings without endothelium usually gave either no increase in active force over the same time, or developed less than 1 g initially and then returned to baseline within approximately 60 min. In endothelium-intact arteries, glibenclamide (3–30 μM) caused concentration-dependent relaxation of the developed spontaneous tone with total relaxation in 3 out of 7 experiments (Figure 1a). In two more experiments in which rings also developed spontaneous force, glibenclamide up to 30 μM failed to cause any relaxation, whilst in the remaining two experiments this concentration of glibenclamide caused less than 50% relaxation. Glibenclamide (3–30 μM) completely relaxed both endothelium-intact and -denuded rings of artery precontracted with U46619 (Figure 1b). The vehicle control (methanol) did not relax spontaneous force in any of these experiments.

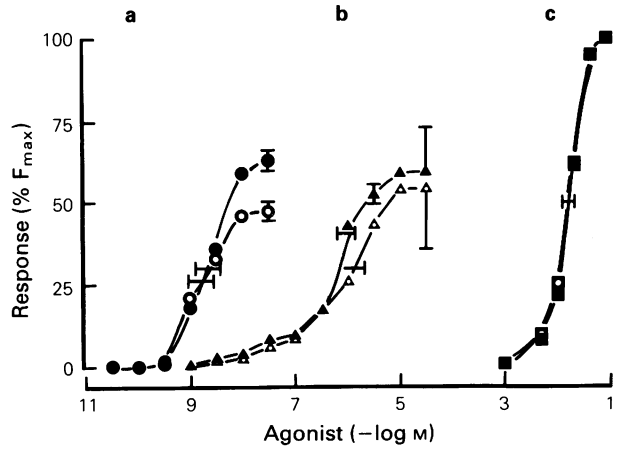

Figure 6 Concentration-contraction curves for (a) endothelin-1, (b) noradrenaline and (c) KCl in the absence (open symbols) and presence (closed symbols) of 30 μM glibenclamide. The noradrenaline curves were constructed in the presence of propranolol (1 μM) in endothelium-denuded rings. The data are expressed as described in Figure 2. The control EC_{50} values (−log M) for endothelin-1, noradrenaline and KCl were, respectively, 8.80 ± 0.24, 5.89 ± 0.19 and 1.80 ± 0.09 (n = 3). The corresponding maximum responses (% F_{max}) for endothelin-1 and noradrenaline were, respectively, 48 ± 7 and 55 ± 19. The corresponding maximum response (g) for KCl was 26 ± 5. Glibenclamide (30 μM) did not significantly alter either the EC_{50} or the maximum response for any of the agonists.

The effect of glibenclamide on the cumulative concentration-contraction curve to U46619 is shown in Figure 2. Increasing concentrations of glibenclamide (1–30 μM) caused a progressive decrease in the sensitivity (EC_{50}) of U46619 but did not significantly alter the maximum response (F_{max}). Schild regression analysis of the effect of glibenclamide on U46619 gave a pK_B of 6.18 (95% confidence limits 5.42 and 7.08) with a slope of the regression line of 1.07 (95% confidence limits 0.78 and 1.34, Figure 3). With the interactive technique, the reciprocal logistic model could be fitted with parallel slopes (F for individual slopes = 0.37, d.f. 4, 219) giving a pK_B of 6.39 (95% confidence limits 6.25, 6.60) with no significant departure from competitivity (n = 1.003, where the parameter n is equivalent to the slope in the Schild plot; see Stone & Angus, 1978). The 'Clark' plot of log A against log (B + K_B) (Figure 4) indicated that all points lay within the 95% confidence limits of the competitive model (indicated by the error bars). Further, there was no evidence of any significant departure of the control point from the competitive model indicating that the direct effect of glibenclamide on resting tone did not interfere with the estimation of the pK_B.

Another sulphonylurea compound, tolbutamide (3 and 30 μM), structurally related to glibenclamide, caused a small but not significant ($P > 0.05$, unpaired t test) decrease in sensitivity to U46619 without affecting the maximal contraction (Figure 5). Glibenclamide (30 μM) had no significant effect on either the EC_{50} or maximal contraction for endothelin-1, noradrenaline or KCl (Figure 6).

Discussion

The hypoglycaemic drug glibenclamide has been reported to be one of the most potent inhibitors of cromakalim-activated K^+ channels in a variety of smooth muscle preparations (see Introduction for references). It is not known whether the same mechanism of action is involved in both this smooth muscle response and the inhibition of ATP-activated K^+ channels involved in the hypoglycaemic response. There is, however, a marked discrepancy between the antagonist potencies for glibenclamide at the cromakalim-activated ATP-regulated K^+ channels in the pancreatic acinar cell (Schmid-Antomarchi *et al.*, 1987; Zünkler *et al.*, 1988; but see Matthews & Shotton, 1984) and the relaxation response to cromakalim in smooth muscle (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze, 1989a,b; Winquist *et al.*, 1989). Regardless of this discrepancy, our data indicate another, previously unreported, property of glibenclamide. The results demonstrate that glibenclamide competitively inhibits the contractile response to a thromboxane A_2 analogue, U46619, in the dog isolated coronary artery. Our estimates of the pK_B for glibenclamide at this vascular thromboxane A_2 receptor were 6.18 and 6.39 by Schild analysis and interactive analysis respectively.

The concern that any glibenclamide-sensitive endogenous thromboxane A_2 may have interfered with the pK_B estimate by the Schild regression method was not supported by the result from the interactive method of analysis. Presumably, basal thromboxane A_2 generation did not occur in all tissues in this assay, a conclusion supported by the preliminary tests with glibenclamide on spontaneously developed force. We have observed similar variability in the effect of a specific thromboxane A_2 receptor antagonist, GR32191 (Lumley *et al.*, 1989) on spontaneously developed force in the dog coronary artery (Cocks & Angus, unpublished data).

In the rat portal vein, glibenclamide (3 μM) caused a 10 fold rightward shift of the cromakalim concentration-relaxation

curve (Winquist *et al.*, 1989) to give an estimated pK_B (assuming competitivity) of 6.48, very similar to our estimate at the thromboxane receptor. Other reports of pK_B values of glibenclamide at the 'cromakalim' receptor in the rat aorta were 6.95 (Cavero *et al.*, 1989) and approximately 7 (calculated from data of Buckingham *et al.*, 1989). In the guinea-pig isolated pulmonary artery, Eltze (1989b) reported similar pK_B values of 7.2 for glibenclamide against three vasorelaxants, cromakalim, pinacidil and RP 49356. In each case the inhibition was competitive. He also demonstrated competitive antagonism by glibenclamide ($pK_B = 7.2$) of the cromakalim-mediated inhibition of twitch contractions in the rabbit vas deferens (Eltze, 1989a). Taken together, these studies clearly indicate that concentrations of glibenclamide in the range 0.1–10 μM that are necessary to antagonize the cromakalim-induced relaxation of smooth muscle fall within the range of the antagonist activity of glibenclamide at the thromboxane A_2 receptor.

In conclusion, we have shown that glibenclamide has an additional novel property of being a relatively potent and selective antagonist of thromboxane A_2 receptors. This action of glibenclamide should be considered in studies on smooth muscle K^+ channels, particularly if a contracting agonist like U46619 is used or if there is spontaneous tone induced presumably by endogenous thromboxane A_2.

This work was supported by an Institute Grant from the National Health and Medical Research Council of Australia. We thank Hoechst Australia for the kind gift of glibenclamide and Clara Chan and Peter Coles for assistance in the manuscript preparation.

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(Received October 26, 1989
Revised February 12, 1990
Accepted February 16, 1990)