Insulin Release from the Perfused Rat Pancreas

MODE OF ACTION OF TOLBUTAMIDE

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Dextran-linked tolbutamide causes first-phase insulin release when perfused through the isolated rat pancreas. The linked sulphonylurea is also able to stimulate the membranal adenylate cyclase present in mouse islets. These facts make it highly likely that sulphonylureas exert their pharmacological action by interaction with the plasma membrane. Their action may be mediated via the adenylate cyclase enzyme.

It has previously been shown that various compounds are capable of stimulating the adenylate cyclase of the β -cell (Atkins & Matty, 1971; Davis & Lazarus, 1972). It has been suggested that sulphonylureas, including tolbutamide and glibenclamide, exert their effects without penetrating the β -cell (Hellman *et al.*, 1971, 1973*a,b*). The present set of experiments was designed to test this hypothesis and to try to ascertain whether the adenylate cyclase is stimulated by sulphonylurea.

Materials and Methods

1-Ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride and Dextran type 200 (average mol.wt. 2×10^6) were supplied by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Adipic acid monomethyl ester and 1,8-diamino-octane were obtained from Ralph N. Emanuel Ltd., London S.E.1, U.K. Toluene-*p*-sulphonamide and CNBr were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Insulin-binding reagent was from Wellcome Research Laboratories, Beckenham, Kent, U.K., and iodinated insulin was from The Radiochemical Centre, Amersham, Bucks., U.K. Thionyl chloride and sodium tolbutamide were supplied by Burroughs Wellcome and Co., London N.W.1, U.K. All other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Preparation of dextran-linked sulphonylurea

Sulphonylurea was linked to dextran as shown in Scheme 1. As a control phenylacetic acid was linked to amino-octyl-dextran (step 9, Scheme 1) in a manner similar to that described for dextran-linked sulphonylurea.

Pancreas perfusions

The method used was that of Grodsky *et al.* (1967) except that Gey and Gey's buffer containing 4% dextran was substituted for the original buffer (Gey & Gey, 1936). Fractions (5ml) were collected at 1 min intervals. The following parameters were monitored continuously throughout the experiment: constancy of buffer flow, 5ml/min; perfusion pressure between 8 and 10.7kPa (60–80mmHg); temperature of the preparation and pH of the buffer. The preparation produced a biphasic response curve when stimulated with glucose.

Adenylate cyclase assay

The assay for adenylate cyclase was as described by Davis & Lazarus (1972). The assay was carried out in the presence of 8mM-theophylline and 2mM-cyclic AMP. Chromatography of cyclic AMP was on Gilman I.T.L.C.-type silica-gel-impregnated glass-fibre sheets, developed for 50min in the ascending direction with propan-2-ol-ethyl acetate-8.5M-NH₃ (60:89:51, by vol.).

Sulphonylurea assay

The material was assayed in the range of 0–400 μ g/ml by reaction with fluorodinitrobenzene in water-saturated amyl acetate. A portion (1 ml) of sample or standard was acidified with 0.4ml of 1M-HCl. Then 5ml of water-saturated amyl acetate was added and the tube shaken well for 4min. Approx. 10mg of activated charcoal was then added to each tube and, after shaking, centrifuged. A portion of amyl acetate (2ml) was then treated with 1ml of fluorodinitrobenzene reagent (0.1% in water-saturated amyl acetate) by heating in tubes plugged with cotton wool

Scheme 1. *Synthesis of dextran-linked sulphonylurea and dextran-linked phenylacetic acid*

in a boiling-water bath for 15min. The tubes were cooled and the absorbance was read at 360nm.

Assay of free sulphonylurea in linked preparation

The following method was used to check the amount of free material present before perfusion. Dextran-linked sulphonylurea was made up in concentrations equal to that used in the pancreas perfusion (100 or 200 μg of sulphonylurea), and the high-

molecular-weight material was removed from the low-molecular-weight material by Amicon filtration (UM 20E). The low-molecular-weight fraction was concentrated by freeze-drying and then reconstituted in 1ml of water. The fraction was concentrated some 50-fold. The presence of sulphonylurea was then assayed as described above.

It was also necessary to determine whether or not any free material was present after perfusion. The tubes that contained linked sulphonylurea after per-

fusion were pooled, the dextran was removed by Amicon filtration, and the low-molecular-weight fraction concentrated by freeze-drying. The fraction was concentrated 25-fold by this method and sulphonylurea was assayed as described above. The

concentration and assay system described above gave 85% recovery of known amounts of added free material.

The assay procedure was able to detect the equivalent of 2.5 μg of free sulphonylurea/ml. The lowest

Fig. 1. First-phase insulin release obtained with free sulphonylurea concentrations of 200 $\mu\text{g/ml}$ (a) and 5 $\mu\text{g/ml}$ (b)

The shaded area shows the period during which the compound was perfused.

Fig. 2. First-phase insulin release obtained with dextran-linked sulphonylurea concentrations of 100 $\mu\text{g/ml}$ (a) and 200 $\mu\text{g/ml}$ (b)

The shaded area shows the period during which the compound was perfused.

concentration of sulphonylurea required to produce stimulation of insulin release was determined by perfusing the pancreas with increasing concentrations of sulphonylurea.

Results

The pancreas was perfused with 1, 2, 3, 4 and 5 μg of free sulphonylurea/ml. Stimulation of insulin release was only obtained with 5 $\mu\text{g}/\text{ml}$. Fig. 1 shows the results obtained with free tolbutamide concentrations of 5 and 200 $\mu\text{g}/\text{ml}$.

Fig. 2 shows that when dextran-linked sulphonylurea was perfused in concentrations of free sulphonylurea equivalent to either 200 or 100 $\mu\text{g}/\text{ml}$, the typical first-phase insulin-secretion response was obtained. Lower concentrations were not used. When the tubes containing the linked sulphonylurea were pooled, filtered, concentrated and then assayed for free material none was detected. When 5 μg of free sulphonylurea/ml was added to the tubes which contained the linked sulphonylurea and then subjected to the same procedure, the free sulphonylurea was easily detected. When 2.5 μg of free sulphonylurea/ml was added to the linked compound, this amount of free sulphonylurea was also easily detected. Thus it seems safe to postulate that there was less than 2.5 μg of free sulphonylurea/ml present in the linked sulphonylurea, this concentration being much too small to cause insulin release. However, it must be emphasized that at no time were we able to detect free sulphonylurea in the preparation.

Dextran-linked phenylacetic acid was without effect at concentrations of free phenylacetic acid equivalent to 200 or 100 $\mu\text{g}/\text{ml}$. The amounts of

phenylacetic acid and sulphonylurea linked to the dextran were similar, i.e. 100 $\mu\text{g}/\text{mg}$ of dextran.

Fig. 3 shows the effect of sulphonylurea on the adenylate cyclase enzyme. The assay was linear for up to 20 min. The stimulation of the adenylate cyclase was concentration-dependent and approx. 100% stimulation was obtained by the use of 13.5 mg of sulphonylurea/ml (50 mM).

Fig. 4 shows the effect of 50 mM-sulphonylurea on the plot of v against s for the adenylate cyclase. The enzyme had maximal activity at 0.6 mM-ATP. The kinetics followed a sigmoid course, and the concentration of ATP at $\frac{1}{2}V_{\text{max}}$ ($s_{0.5}$) was 0.25 mM. The sulphonylurea stimulated at all concentrations of ATP. At 0.6 mM-ATP this stimulation was approximately 150-fold greater than without added sulphonylurea. The $\frac{1}{2}V_{\text{max}}$ ($s_{0.5}$) for ATP was 0.27 mM.

Table 1 shows that dextran-linked sulphonylurea also stimulates the adenylate cyclase. Dextran-linked phenylacetic acid is without effect.

Discussion

The lowest concentration of free sulphonylurea that was able to cause insulin secretion was 5 $\mu\text{g}/\text{ml}$. This amount after concentration could easily be detected in the assay procedure. Since over 85% of the perfused material was recovered in the perfusate it is unlikely that free sulphonylurea was being sequestered in the pancreas. The lowest concentration of free sulphonylurea detectable was 2.5 $\mu\text{g}/\text{ml}$, an amount that was without effect on the pancreas. When the dextran-linked sulphonylurea was tested for free material both before and after perfusion none

Fig. 3. Effect of sulphonylurea on adenylate cyclase activity

(a) Time-course for adenylate cyclase activity when incubated alone (●), or with (■) 5 mM-, (▲) 10 mM- or (○) 50 mM-sulphonylurea. (b) Effect of sulphonylurea on adenylate cyclase activity.

Fig. 4. Plot of v against s for the adenylate cyclase enzyme

●, Enzyme alone; ▲, 50mM-sulphonylurea. At $0.5V_{\max}$, ($s_{0.5}$) the concentration of ATP is 0.25 mM.

was detected. When free sulphonylurea at concentrations equivalent to 2.5 and 5.0 $\mu\text{g/ml}$ was added to the linked compound and then tested for, free sulphonylurea was easily detected.

The use of dextran-linked phenylacetic acid, prepared under exactly the same conditions, eliminated any effects that may have been introduced by the method of linkage. It also demonstrated that insulin release was dependent on the presence of the sulphonylurea moiety and not on side chains etc.

Sulphonylurea stimulates the adenylate cyclase of mouse islets (Figs. 3 and 4), as does the linked sulphonylurea. This stimulation is obtained in the presence of 8mM-theophylline, which causes 50% inhibition of the phosphodiesterase, and 2mM-cyclic AMP, a concentration that is 200-fold higher than the K_m for the uninhibited enzyme (Bowen & Lazarus, 1973). Under these conditions it is unlikely that the effect of sulphonylurea is mediated via an inhibitory action on the phosphodiesterase. As Fig. 4 shows, the effect of sulphonylurea appears to be on $V_{\max}$. [the $\frac{1}{2}V_{\max}$, ($s_{0.5}$) concentration of ATP is not altered]. The smallest concentration of sulphonylurea that could stimulate the adenylate cyclase was 0.5mM (see Fig. 3). The smallest concentration of sulphonylurea that could cause insulin release in the perfused pancreas was 0.02mM, i.e. a difference of some 25-fold. We are unable to account for this discrepancy. Damage of the tolbutamide receptor during the isolation procedure is possible and could account for the

Table 1. Effect of free and dextran-linked tolbutamide on mouse islet adenylate cyclase

All concentrations at 10mM. This is the concentration of free sulphonylurea that gives $\frac{1}{2}V_{\max}$ stimulation of the mouse adenylate cyclase enzyme. Values are $\pm$ S.E.M. for five experiments.

	Adenylate cyclase activity (pmol of cyclic AMP/min per 100 μg of protein)
Control	97.6 $\pm$ 6.0
Free sulphonylurea	192.5 $\pm$ 16.4
Dextran-linked phenylacetic acid	117.8 $\pm$ 15.1
Dextran-linked sulphonylurea	173.5 $\pm$ 10.3

difference. However, in the absence of more specific data this remains speculative.

Our conclusions that tolbutamide does not have to enter the β -cell for it to exert its pharmacological action are in basic agreement with the work of Hellman *et al.* (1973a,b). These workers obtained their results by using completely different techniques.

It would appear that there are at least two sites of action for the sulphonylureas, i.e. stimulation of the adenylate cyclase (Kuo *et al.*, 1973) and/or inhibition of the phosphodiesterase enzyme (Rosen *et al.*, 1971; Ashcroft *et al.*, 1972). Since it has been shown that tolbutamide does not pass through the β -cell membrane we presume that the phosphodiesterase that is inhibited is the one that is membrane-bound (Davis & Lazarus, 1972). The results in the present paper suggest that the effects of tolbutamide are at least partially mediated by cyclic AMP, but whether this is the sole mechanism of action remains to be clarified.

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