Internalization of ^3H -glibenclamide in pancreatic islet cells

J.-L. Carpentier¹, F. Sawano¹, M. Ravazzola¹ and W.J. Malaisse²

¹ Institute of Histology and Embryology, University Medical Center, Geneva, Switzerland;

² Laboratory of Experimental Medicine Brussels Free University, Brussels, Belgium

Summary. As judged by autoradiographic criteria at the optical level, rat islet cells progressively accumulate ^3H -glibenclamide over 1 to 30 min incubation at 24 °C. The labeled material associates with all endocrine cells, but is preferentially seen within B cells. The total uptake of ^3H -glibenclamide (1.0 $\mu\text{mol/l}$) is little affected by the presence of unlabeled glibenclamide (0.2 mmol/l), but is significantly decreased at 4 °C ($p < 0.05$). At the electron microscopic level, less than 15% of the autoradiographic grains are located at the B cell plasma

membrane and 72–79% of the grains found over the cytoplasm are associated with insulin secretory granules. Such a pattern is observed both after short (1 min) or prolonged (30 min) incubation, and at 4 or 24 °C. It is proposed that the insulinotropic action of glibenclamide is not necessarily attributable to primary events located solely at the cell surface.

Key words: Glibenclamide, morphology, autoradiography, isolated islets.

Hypoglycaemic sulfonylureas stimulate insulin release. Although the molecular determinants of their insulinotropic action remain to be fully elucidated, it is currently assumed that these drugs bind to the B-cell plasma membrane and display little or no ability to enter into the B cell [1]. The existence of specific membrane receptors for sulfonylureas was even postulated [2]. However, the criteria adopted for specific binding are also fulfilled when sulfonylureas interact with phospholipid bilayers in an artificial system [3]. The present ultrastructural study was undertaken to verify whether ^3H -glibenclamide is indeed solely bound to the plasma membrane of islet cells without being internalized into such cells.

Materials and methods

^3H -glibenclamide (31.1 Ci/mmol), tritiated in the cyclohexyl ring, was kindly provided by M.H. Mertens (Hoechst Belgium, Brussels, Belgium) and by Hoechst Switzerland (Zurich, Switzerland). As judged by thin layer chromatography on Silicagel plates, 97–98% of the radioactivity in the preparation of ^3H -glibenclamide gave the same reference as pure glibenclamide.

Groups of 50 islets each, isolated from fed albino breed rats [4], were placed in 100 μl of a Krebs bicarbonate-buffered medium [5] containing D-glucose (2.8 mmol/l), ^3H -glibenclamide (1.0 $\mu\text{mol/l}$), bovine serum albumin (2.5 mg/ml), and, when required, unlabeled glibenclamide (0.2 mmol/l) obtained from a stock solution 17 mmol/l in a mixture of ethanol and NaOH (0.1 mol/l). The islets were incubated for 1 to 30 min at 24 °C or for 60 min at 4 °C, washed once with nonradioactive buffer, fixed in 2% glutaraldehyde in phosphate buffer (0.1 mol/l, pH 7.4) for 30 min, and processed for autoradiography, as described elsewhere [6].

The total uptake of radioactivity was judged from semi-thin sections, using a 3000 magnification, and expressed as the number of grains per μm^2 . For each experimental condition, 4 to 11 separate pictures were examined. The subcellular location of silver grains was judged in thin section, photographed using a 11,000 magnification, and calibrated with a reference grid (2160 lines/mm) (Ernest F. Fulham Inc., Schenectady, NY, USA). For each time point a mean of

180 grains was photographed in each experiment. Quantitative determinations were carried out either on negatives enlarged 3.75 times (final magnification: $\times 41,250$) or positive prints enlarged 3 times (final magnification: $\times 33,000$). The association of the labeled material with the plasma membrane of B cells was assessed quantitatively by the method of Salpeter et al. [7]. The percentage of total number of grains was plotted as a function of the distance between the grain center and the closest plasma membranes as previously described [6]. Grains located within 250 nm of the plasma membrane were assumed to represent plasma membrane-bound ^3H -glibenclamide; the other grains were assumed to represent internalized ^3H -glibenclamide. Grains located within 250 nm of the secretory granule limiting membrane were assumed to represent granule-bound ^3H -glibenclamide and were expressed in percent of the total number of cytoplasm-associated grains.

The analysis of the association of the labeled material with the various endocrine cells was performed on a total of 8 different islets incubated for 10 or 30 min in the presence of ^3H -glibenclamide. The number of B and non-B endocrine cell encountered in the islet sections was recorded together with the cell association of the autoradiographic grains. On the basis of these observations, the grain density over B and non-B endocrine cells were calculated. A total of 1053 autoradiographic grains and 2210 endocrine cell sections were analyzed by this method.

In order to characterize the radioactive material associated with the islets, four groups of 60 islets each were incubated for 30 min at 24 °C, and then washed and fixed as described above. After removal of glutaraldehyde, the islet pellet was extracted with a mixture (1.0 ml) of toluene and butanol (7:3, v:v). The extracts, which contained 93% of the total radioactivity associated with the islet pellet after fixation, were pooled, dried, reconstituted in the same organic mixture and run on Silicagel plates (60F254; Merck, Darmstadt, FRG) in chloroform/cyclohexane/ethanol/acetic acid (9:9:1:1; v:v:v:v).

Statistical analysis

Statistical significance was determined with the Student's t-test.

Results

Nature of the cell-associated radioactivity

After 30 min incubation at 24 °C, the uptake of ^3H -glibenclamide (1.0 $\mu\text{mol/l}$) averaged 29.7 ± 0.6 fmol/islet

Fig. 1. Time course for ^3H -glibenclamide uptake by islet cells incubated at 24°C . Mean values ($\pm$ SEM) refer to 6–11 individual determinations

Fig. 2. Portion of the cytoplasm of a B cell from an islet of Langerhans incubated for 30 min in the presence of ^3H -glibenclamide and processed for EM autoradiography. Autoradiographic grains are seen overlying the limiting membrane of β -granules. $\times 22,400$

Table 1. Autoradiographic grain density over B and non-B endocrine cells of the islets of Langerhans incubated in the presence of ^3H -glibenclamide

	Grain density (no. grains per cell)
B cells ($n=8$)	0.55 ± 0.15
Non-B endocrine cells ($n=8$)	0.23 ± 0.06

$p < 0.01$

Table 2. Association of autoradiographic grains to the plasma membrane and to secretory granules in B cells of islets exposed to ^3H -glibenclamide (1.0 mmol/l)

Time (min)	Temperature ($^\circ\text{C}$)	Plasma membrane (% of total)	Granules (% of internalized)
1	24	11.4	79.0
5	24	12.7	71.7
30	24	9.4	76.9
60	4	14.2	76.7

($n=4$). After further fixation for 30 min in glutaraldehyde, more than 90% of the radioactive material extracted from the islet pellet by a mixture of toluene and butanol yielded the same chromatographic reference as ^3H -glibenclamide.

Morphological analysis of cell-associated radioactivity

Figure 1 illustrates the time course for ^3H -glibenclamide uptake by the islet cells incubated at 24°C . Within 30 min, a steady-state value for ^3H -glibenclamide uptake was not yet reached. At min 30 of incubation, the uptake of ^3H -glibenclamide ($1.0 \mu\text{mol/l}$) was little affected by the presence of a high concentration of unlabeled glibenclamide (0.2 mmol/l), averaging $85.5 \pm 17.3\%$ ($n=8$) of the mean value found in the sole presence of labeled sulfonylurea. The uptake was significantly decreased, however, at 4°C . Thus, even after 60 min incubation at 4°C , the uptake of ^3H -glibenclamide ($1.0 \mu\text{mol/l}$) averaged no more than $174 \pm 27 \text{ grains}/\mu\text{m}^2$ ($n=8$), as distinct ($p < 0.05$) from $283 \pm 36 \text{ grains}/\mu\text{m}^2$ ($n=11$) after only 30 min incubation at 24°C .

Autoradiographic grains were observed over all islet cells but as judged by the grain density over B cells versus grain density over non-B endocrine cells the labeled material preferentially associated with B cells (Table 1). In the B cells, 9–14% of the grains were located at the plasma membrane (Table 2). The remaining grains, assumed to represent internalized ^3H -glibenclamide, were located mostly at the level of secretory granules (Fig. 2). Thus, relative to internalized material, 72–79% of the grains were within 250 nm of the granule-limiting membrane. Figure 3 provides quantitative information on the distribution of grains in the vicinity of the plasma membrane and secretory granule limiting membrane respectively. It should be underlined that the pattern of distribution was little affected by either the length of exposure to ^3H -glibenclamide (1 to 30 min) or temperature of incubation (4 versus 24°C).

Discussion

The results illustrated in Figure 1 are in good agreement with those recently reported by Hellman et al. [8], indicating that glibenclamide accumulates progressively in islets from ob/ob mice incubated at 37°C in the presence of $20 \mu\text{mol/l}$ ^3H -glibenclamide. The failure of unlabeled glibenclamide to cause a sizeable fall in ^3H -glibenclamide uptake is also in good agreement with a prior observation indicating that the islet uptake of ^{14}C -labeled glibenclamide is proportional to the concentration of the drug in the $10 \mu\text{mol/l}$ to 0.1 mmol/l range [9].

The novel information provided by this study concerns the cellular and subcellular location of ^3H -glibenclamide. First, with respect to the cellular location the labeled material preferentially associates with B cells.

Fig. 3. Frequency distribution of autoradiographic grains in the vicinity of the plasma membrane (left) and secretory granule limiting membrane (right) in B cells of islets exposed to ^3H -glibenclamide. The length and temperature of incubation are indicated in each case

Second, as judged by autoradiographic criteria, less than 15% of the radioactive grains were associated with the B cell plasma membrane, the major fraction of radioactive material being internalized. Of the latter fraction, 72–79% were associated with insulin secretory granules. This pattern of distribution was comparable after short (1 min) or prolonged (30 min) incubation at 24°C. In the latter case (30–60 min incubation), the distribution pattern was also similar at 4 and 24°C respectively, although the total uptake of ^3H -glibenclamide was decreased at the low temperature. Several modalities could be considered for the rapid internalization of ^3H -glibenclamide in islet cells [10]. Whatever the precise modality, the accumulation of ^3H -glibenclamide in association with secretory granules raises the possibility that these organelles represent a target for the biophysical effects of this hypoglycaemic sulfonylureas, e.g. the nucleophilic interaction with membrane-located sulfhydryl groups [1] or the formation of lipophilic ion complexes [11]. It seems premature to ascribe the insulin-

tropic action of glibenclamide solely [1] to primary events specifically located at the cell surface. It is, nevertheless, a reassuring finding that ^3H -glibenclamide is internalized to a rather large extent. We have already indicated [3] that the uptake of glibenclamide (0.1 mmol/l) by intact islets would correspond to a sulfonylurea/phospholipid molar ratio close to unity if the hypoglycaemic sulfonylurea were to be located solely in the plasma membrane.

Acknowledgements. We thank Dr. L. Orci for helpful discussions and critical reading of the manuscript. We are indebted to Drs. F. Malaisse-Lagae and F. Gomez for help in these experiments, and to Ms G. Berthet and M. Muller for skilled technical and typographical assistance. This investigation was supported in part by a grant no. 3.460.83 from the Swiss National Science Foundation and a grant from the Belgian Ministry of Scientific Policy.

References

1. Gylfe E, Hellman B, Sehlin J, Taljedal I-B (1984) Interaction of sulfonylurea with pancreatic B-cell. *Experientia* 40: 1126–1134
2. Kaubisch N, Hammer R, Wollheim C, Renold AE, Offord RE (1982) Specific receptors for sulfonylureas in brain and in a β -cell tumor of the rat. *Biochem Pharmacol* 31: 1171–1174
3. Deleers M, Malaisse WJ (1984) Binding of hypoglycaemic sulfonylureas to an artificial phospholipid bilayer. *Diabetologia* 25: 55–59
4. Lacy PE, Kostianovsky M (1967) Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16: 35–39
5. Malaisse-Lagae F, Malaisse WJ (1983) Insulin release by pancreatic islets. In: Lerner J, Pohl SJ (eds) *Methods in diabetes research*, vol I, part B. John Wiley, New York, pp 147–152
6. Carpentier J-L, Gorden P, Amherdt M, Van Obberghen E, Kahn CR, Orci L (1978) ^{125}I -insulin binding to cultured human lymphocytes: initial localization and fate of hormone determined by quantitative electron microscopic autoradiography. *J Clin Invest* 61: 1057–1070
7. Salpeter MM, Fertuck HC, Salpeter EE (1977) Resolution in electron microscope autoradiography. III. Iodine-125. The effect of heavy metal staining and a reassessment of critical parameters. *J Cell Biol* 72: 161–173
8. Hellman B, Sehlin J, Taljedal I-B (1984) Glibenclamide is exceptional among hypoglycaemic sulfonylureas in accumulating progressively in β -cell-rich pancreatic islets. *Acta Endocrinol (Kbh)* 105: 389–390
9. Hellman B, Sehlin J, Taljedal I-B (1973) The pancreatic β -cell recognition of insulin secretagogues. IV. Islet uptake of sulfonylureas. *Diabetologia* 9: 210–216
10. Steinman RM, Mellman IS, Muller WA, Cohn ZA (1983) Endocytosis and the recycling of plasma membrane. *J Cell Biol* 96: 1–27
11. Malaisse WJ, Hubinont C, Lebrun P, Herchuelz A, Couturier E, Deleers M, Malaisse-Lagae F, Sener A (1983) Mode of action of hypoglycaemic sulfonylureas in the pancreatic B-cell: coinciding and conflicting views. In: *Clinical and pharmacological activities of sulfonylurea drugs*. Serrano-Rios M, Krahil LP (eds) *Excerpta Medica*, Amsterdam, pp 24–38

Received: 5 November 1985
and in revised form: 17 January 1986

Dr. J.-L. Carpentier
Institut d'Histologie et d'Embryologie
CMU
1, rue Michel Servet
CH-1211 Genève 4
Switzerland