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In vitro and *in vivo* effects of new insulin releasing agents

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Abstract

The present study aimed at characterizing *in vitro* and *in vivo* the effects of BM 208 (*N*-[4-(5-chloro-2-methoxybenzamidoethyl)-benzenesulfonyl]-*N'*-cyano-*N''*-cyclohexylguanidine) and BM 225 (1-[4-(5-chloro-2-methoxybenzamidoethyl)benzene sulfonamido]-1-cyclohexylamino-2-nitroethylene); two new isosteres of the hypoglycemic sulfonylurea glibenclamide. In rat pancreatic islets perifused at close to normal (8.3 mM) D-glucose concentration, both BM 208 and BM 225 (10 and 25 μM) increased ^{45}Ca outflow and insulin release. The compounds did not affect the ^{45}Ca outflow rate from islets exposed to Ca^{2+}-free media. In single pancreatic islet cells loaded with the fluorescent Ca^{2+} indicator fura-2 and incubated in the presence of 8.3 mM glucose, BM 208 and BM 225 raised the $[Ca^{2+}]_i$. All these findings indicate that, in islet cells exposed to a physiological concentration of D-glucose, the secretory capacity of the new glibenclamide isosteres is related to a facilitation of Ca^{2+} entry. The potency and duration of action of BM 225 was, however, more pronounced than that of BM 208. Successive additions of BM 208 provoked repeated increments in ^{45}Ca outflow and insulin release, without evidence of tachyphylaxis. Lastly, intraperitoneal injection of BM 208 and BM 225 to fed rats lowered plasma glucose concentration in a dose-dependent manner. BM 225 was more potent and acting faster than BM 208. Our results indicate that appropriate structural modification can generate isosteres of glibenclamide with different features and activity profiles.

Keywords: BM 208; BM 225; Pancreatic B cells; Insulin release; Glycaemia

1. Introduction

Type II diabetes mellitus, the most common form of diabetes, is usually treated by a non-pharmacologic intervention combined, upon failure, with oral antidiabetic drugs [1]. Various pharmacological compounds can be used to improve glucose homeostasis but hypoglycaemic sulfonylureas have an established place in treatment of type II diabetic patients [1,2]. Their hypoglycaemic action is primarily mediated through a direct stimulation of insulin release [1,3,4]. The main mechanism responsible for the insulin releasing capacity of hypoglycaemic sulfonylureas implicates the closure of ATP-sensitive K^+ (K_{ATP}) channels, heteromultimers composed of sulfonylurea receptor (SUR_1) and Kir 6.2 subunits [5], leading to cell depolarization, Ca^{2+} entry and exocytosis of secretory granules [3,4,6].

In the search for new antidiabetic compounds exhibiting differences in potency, in rate of onset or duration of action, we have recently developed original sulfonylcyanoguanidines and sulfonamidonitroethylenes [7,8]. Among the different compounds synthesized, BM 208 and BM 225 can be considered as isosteres of glibenclamide and preliminary studies with intact rat pancreatic islets indicated that both compounds acted as potent insulin releasing agents [8]. Like other hypoglycaemic sulfonylureas, their insulinotropic action was attributable in part to the inhibition of K_{ATP} channels located at the plasma membrane of pancreatic B cells [9].

The initial aim of the present study was to examine the cationic and secretory response to BM 208 and BM 225 in pancreatic islets exposed to close to normal (8.3 mM) D-glucose concentration. Emphasis was placed on the pattern of responses after the administration of both

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Abbreviations: FOR, fractional outflow rate; K_{ATP} channels, ATP-sensitive K^+ channels $[Ca^{2+}]_i$ = cytosolic Ca^{2+} concentration; BM 208, *N*-[4-(5-chloro-2-methoxybenzamidoethyl)benzenesulfonyl]-*N'*-cyano-*N''*-cyclohexylguanidine; BM 225, 1-[4-(5-chloro-2-methoxybenzamidoethyl)benzene sulfonamido]-1-cyclohexylamino-2-nitroethylene.

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compounds. The study was also undertaken to assess the hypoglycaemic activity of BM 208 and BM 225 in fed rats.

2. Materials and methods

2.1. Measurements of ^{45}Ca outflow and insulin release from perifused pancreatic islets

Experiments were performed with pancreatic islets isolated by the collagenase method from fed Wistar albino rats (Proefdierencentrum KUL).

The methods used to measure ^{45}Ca outflow and insulin release from perifused islets have been described previously [10–12]. Groups of 100 islets were incubated for 60 min in a bicarbonate buffered medium (in mM: NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24) containing 16.7 mM glucose and ^{45}Ca (0.02–0.04 mM; 100 μCi/mL). After incubation, the islets were washed four times with a non-radioactive medium and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL/min). From the 31 to 90 min of perifusion, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.5 mL) was used for scintillation counting while the remainder was stored at −20° for insulin radioimmunoassay [13]. At the end of the perifusion, the radioactive content of the islets was also determined. The outflow of ^{45}Ca (cpm/min) was expressed as a fractional outflow rate (percent of instantaneous islet content/min; FOR).

2.2. Measurements of fura-2 fluorescence from single rat pancreatic islet cells

Pancreatic islets were disrupted in a Ca^{2+}-deprived medium and then centrifuged to remove debris and dead cells. Cells were seeded onto glass coverslips and maintained in tissue culture for 72 hr before use. Islet cells were cultured in RPMI 1640 culture medium (Life Technologies) supplemented with 10% (v/v) newborn calf serum and containing glutamine (2.3 mM), glucose (16.7 mM), penicillin G (100 IU/mL) and streptomycin (100 μg/mL). The cells were then incubated with fura-2 AM (2 μM) (Molecular Probes) for 1 hr and, after washing, the coverslips with the cells were mounted as the bottom of an open chamber (1 mL) placed on the stage of the microscope. The medium used to perifuse the cells contained (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, glucose 2.8 and was gassed with O_2 (95%)/CO_2 (5%). Fura-2 fluorescence of single loaded cells (selected on the basis of their larger size) [14] was measured by use of dual-excitation microfluorimetry with a Spex photometric system (Optilas, Alphen aan den Rijn). The excitation wavelengths (340 and 380 nm) were alternated at the frequency of 1 Hz. The emission wavelength was set at 510 nm. $[Ca^{2+}]_i$ was calculated as previously described [10]. Individual experiments were repeated at least 3–4 times, on different cell populations.

2.3. Measurements of glycaemia

Animals (adult fed Wistar albino rats) were allowed to settle in for at least 3 days in the laboratory before use. They had free access to water and received a standard pellet diet. Conscious rats were placed for 60 min prior blood sampling and throughout the duration of the study in a small cabinet. Drugs were dissolved in a sterile physiological solution (NaCl 0.9%) supplemented with 1 eq. NaOH to form the sodium salt. At zero time, BM 208 or BM 225 were administered intraperitoneally (i.p.) at a dosage of 20 and 50 mg/kg. Control animals received an equivalent volume of NaCl 0.9% supplemented with NaOH; 5 mL/kg body weight. Blood was taken from the tail at time—5, 30, 60, 120 and 180 min. Blood glucose concentration was measured, in duplicate, using a reagent strip in combination with a glucometer (ACCU-TREND, Boehringer, Mannheim).

2.4. Drugs

In some *in vitro* experiments, extracellular Ca^{2+} was eliminated by the omission of $CaCl_2$ from the physiological medium and the addition of 0.5 mM ethylene glycol-bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA; Sigma). According to the experiments, the media also contained glucose (Merck), BM 208 or BM 225. BM 208 and BM 225 were synthesized at the Department of Medicinal Chemistry, Université de Liège, Belgium [7,8]. For the *in vitro* experiments, BM 208 and BM 225 were dissolved in dimethylsulphoxide which was added to both control and test media at final concentrations not exceeding 0.1% (v/v). At this concentration, dimethylsulphoxide fails to affect islet function [10,15].

2.5. Calculations

Results are expressed as means ± SE. The basal value for ^{45}Ca outflow and insulin release was computed from the 40–44, 41–45 or the 71–75 min of perifusion inclusive. The magnitude of the increase in ^{45}Ca outflow or insulin release was estimated in each individual experiment from the integrated outflow of ^{45}Ca or insulin release observed during stimulation (45–68, 46–55 or 76–85 min) after correction for basal value. The statistical significance of differences between mean data was assessed by using Student's *t*-test.

3. Results

3.1. Effects of BM 208 and BM 225 on ^{45}Ca outflow and insulin release

At an insulinotropic concentration of D-glucose, i.e. 8.3 mM, both BM 208 (10 μM) and BM 225 (10 μM)

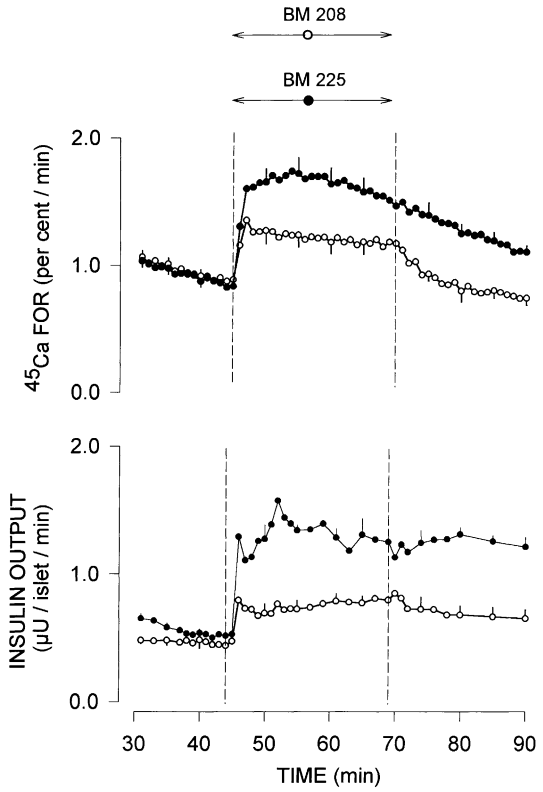

Fig. 1. Effect of BM 208 (○; 10 μM) and BM 225 (●; 10 μM) on ^{45}Ca outflow (upper panel) and insulin release (lower panel) from pancreatic islets perifused throughout in the presence of 8.3 mM glucose and extracellular Ca^{2+}. Mean values ± SE refer to four to six individual experiments.

caused a rapid and sustained stimulation of ^{45}Ca outflow from pancreatic islets perifused in the presence of extracellular Ca^{2+} (Fig. 1, upper panel). The enhancing effect of BM 225 was more marked than that of BM 208. Thus, the integrated outflow of ^{45}Ca observed during exposure to the drugs averaged 0.30 ± 0.05%/min after addition of BM 208 (10 μM) and 0.74 ± 0.07%/min after addition of BM 225 (10 μM) ($P < 0.05$). Moreover, it must be stressed that the stimulatory effect of BM 208 was reversible whilst the ^{45}Ca FOR remained sustained after the removal of BM 225 from the perifusate (Fig. 1, upper panel).

Under the same experimental conditions, both drugs increased insulin output (Fig. 1, lower panel). BM 225 evoked, again a more pronounced ($P < 0.05$) and less reversible secretory response.

Fig. 2 illustrates the effect of 25 μM BM 208 and 25 μM BM 225 on ^{45}Ca outflow and insulin release from islets perifused throughout in the presence of 8.3 mM glucose. When the experiments were conducted in the presence of extracellular Ca^{2+}, both compounds provoked a marked increase in ^{45}Ca outflow (Fig. 2, upper panels). The paired difference in ^{45}Ca FOR before (40–44 min) and during (45–68 min) exposure to 25 μM BM 208 or 25 μM BM 225 averaged 0.45 ± 0.03 and 0.75 ± 0.07%/min, respectively ($P < 0.05$).

To further investigate the effects of BM 208 and BM 225 on ^{45}Ca movements, the same experiments were conducted in the presence of 8.3 mM glucose but absence of extracellular Ca^{2+} (Fig. 2, upper panels). In islets exposed to

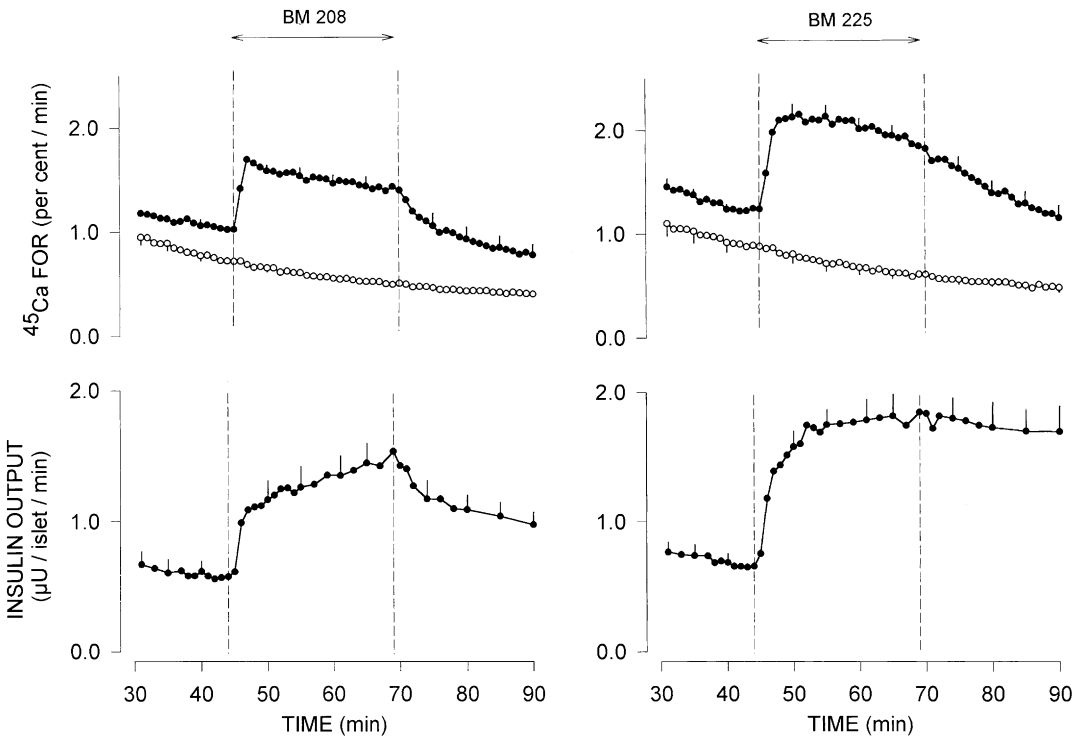

Fig. 2. Effect of BM 208 (25 μM, left panels) and BM 225 (25 μM; right panels) on ^{45}Ca outflow (upper panels) and insulin release (lower panels) from pancreatic islets perifused throughout in the presence of 8.3 mM glucose. Basal media contained Ca^{2+} (●) or were deprived of Ca^{2+} and enriched with EGTA (○). Mean values ± SE refer to four to six individual experiments.

Ca^{2+}-free media, the rate of ^{45}Ca outflow before drug administration was lower ($P < 0.05$ in each case). The addition of 25 μM BM 208 (left panel) or 25 μM BM 225 (right panel) did not affect the ^{45}Ca fractional outflow rate.

In the presence of 8.3 mM glucose and extracellular Ca^{2+} in the perifusing medium, BM 208 (25 μM) and BM 225 (25 μM) elicited an immediate and sustained secretory response (Fig. 2, lower panels). As previously noticed, BM 208 was less effective than BM 225 at increasing insulin release. Indeed, the magnitude of the increment in insulin output averaged 0.67 ± 0.08 μunit per islet/min after addition of BM 208 and 0.99 ± 0.07 μunit per islet/min after addition of BM 225, respectively ($P < 0.05$).

These experiments conducted with a higher concentration of BM 208 or BM 225 further confirm that after long-term exposure (25 min), the cationic and secretory responses to BM 225 were not readily reversible.

3.2. Effects of iterative administrations of BM 208 on ^{45}Ca outflow and insulin release

In the next series of experiments, we characterized the effects of repeated administrations of BM 208 on the cationic and secretory responses of perifused rat pancreatic islets (Fig. 3). In the presence of 8.3 mM glucose in the perifusing medium, the iterative administrations of BM 208 (10 μM) for 10 min each provoked, at the occasion of two successive additions, a rapid and sustained increase in ^{45}Ca outflow (Fig. 3, upper panel). When the intervening resting periods lasted 20 min, the magnitude of the successive increments in ^{45}Ca outflow evoked by BM 208 was similar. The increase in ^{45}Ca FOR averaged $0.32 \pm 0.10\%$/min after the first exposure and $0.29 \pm 0.05\%$/min after the second exposure to BM 208 ($P > 0.05$). Upon each withdrawal of BM 208, a rapid decrease in effluent radioactivity was noticed (Fig. 3, upper panel).

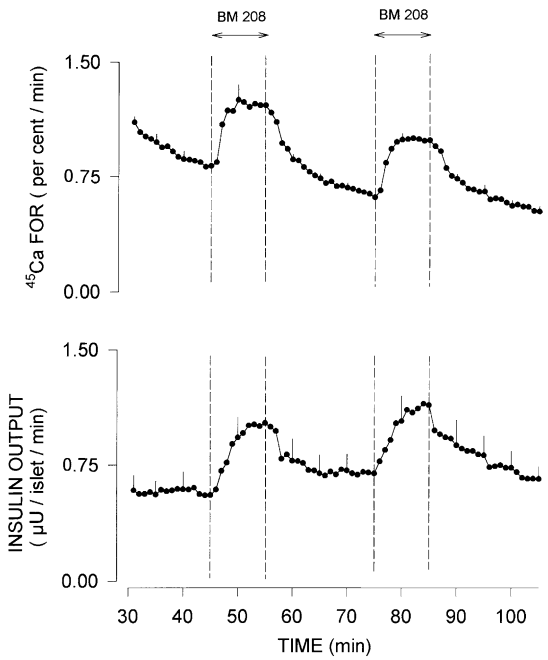

Fig. 3. Effect of iterative administrations of BM 208 (10 μM) on ^{45}Ca outflow (upper panel) and insulin release (lower panel) from pancreatic islets perifused throughout in the presence of 8.3 mM glucose and extracellular Ca^{2+}. Mean values ± SE refer to four individual experiments.

The figure also shows that iterative administrations of BM 208 (10 μM) to islets perifused throughout in the presence of 8.3 mM glucose provoke repeated increments in insulin release (Fig. 3, lower panel). These successive increases in insulin output were rapidly reversible upon removal of BM 208 from the perifusate and displayed comparable magnitude at the occasion of each stimulation. The increments in insulin output above basal values averaged 0.32 ± 0.04 μunit per islet/min during the first exposure to BM 208 and 0.34 ± 0.07 μunit per islet/min during the second administration of the drug ($P > 0.05$).

3.3. Effects of BM 208 and BM 225 on glucose-induced increase in cytosolic-free Ca^{2+} concentration

A rise in the extracellular concentration of glucose from 2.8 to 8.3 mM provoked an initial and transient reduction rapidly followed by a marked increase in cytosolic Ca^{2+} concentration (Fig. 4A–D). In 7 out of 14 cells, the change in extracellular glucose concentration elicited a monophasic increase in $[Ca^{2+}]_i$ (Fig. 4A and B). In the remaining cells, the cytosolic Ca^{2+} response to glucose was biphasic; an initial peak being followed by a more sustained plateau (Fig. 4C and D). Whatever the pattern of the glucose response, the subsequent addition of either BM 208 (25 μM) or BM 225 (25 μM) further increased the $[Ca^{2+}]_i$. The enhancing effect of BM 225, however, was invariably more marked and more sustained.

3.4. Effects of BM 208 and BM 225 on blood glucose level

In control rats receiving the vehicle, the blood glucose level was nearly stable during 180 min (Fig. 5).

The injection of BM 208 provoked a concentration-dependent decrease in glycaemia (Fig. 5, left panel). For the low BM 208 concentration (20 mg/kg), however, the drop in blood glucose was only evident by 120 min ($P < 0.05$). At the 120 min, the blood glucose level amounted to 128.4 ± 4.0 mg/100 mL and 102.1 ± 1.3 mg/100 mL after the administration of 20 and 50 mg/kg BM 208, respectively ($P < 0.05$).

BM 225 also induced dose-dependent reductions in glycaemia. The effects of BM 225 were more rapid and more marked than those elicited by BM 208 (Fig. 5, compare left and right panels). At the 120 min, the glycaemia averaged 81.3 ± 2.5 mg/100 mL after the administration of 20 mg/kg and 68.7 ± 2.0 mg/100 mL after the administration of 50 mg/kg BM 225, respectively ($P < 0.05$).

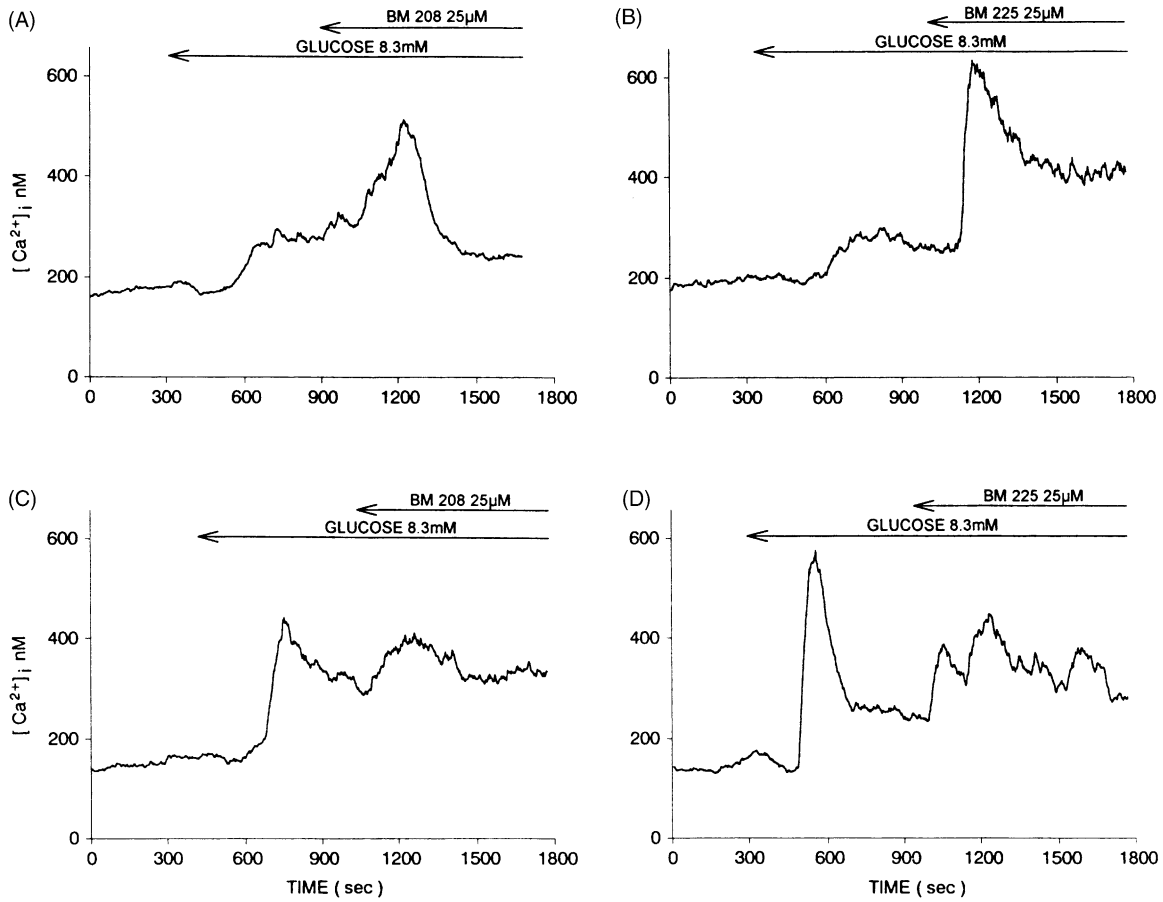

Fig. 4. Effect of a rise in the extracellular glucose concentration from 2.8 to 8.3 mM and the further addition of BM 208 (25 μM, left panels, A and C) or BM 225 (25 μM, right panels, B and D) on the cytosolic Ca^{2+} concentration of single islet cells. Basal media contained extracellular Ca^{2+}. Each graph is a representative experiment conducted on a single cell.

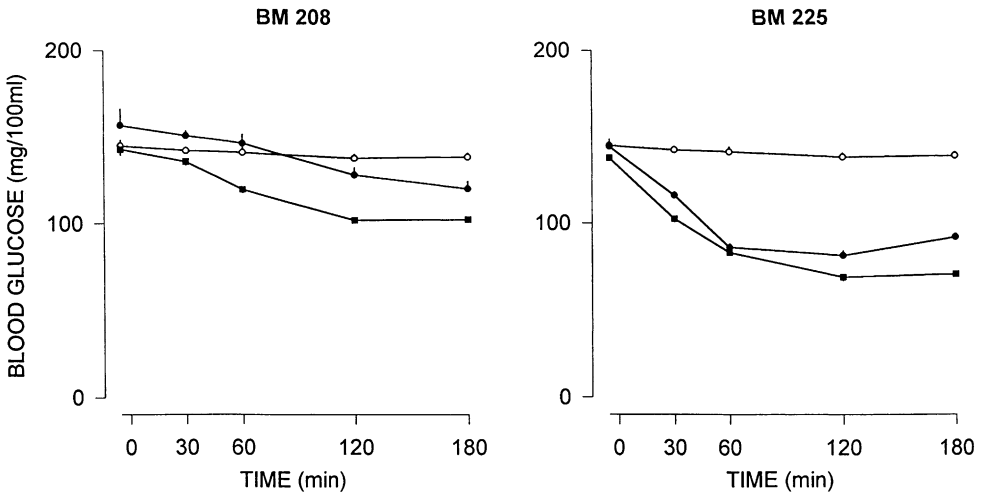

Fig. 5. Time courses for the changes in blood glucose level in fed rats injected intraperitoneally at time zero with BM compounds or the vehicle. Left panel: control (○); BM 208 (●; 20 mg/kg); BM 208 (■; 50 mg/kg). Right panel: control (○); BM 225 (●; 20 mg/kg); BM 225 (■; 50 mg/kg). Mean values ± SE refer to four to seven individual experiments.

4. Discussion

The present *in vitro* data indicate that at close to physiological extracellular D-glucose concentration, i.e. 8.3 mM, BM 208 and BM 225 (Fig. 6) markedly stimulated insulin output from perifused rat pancreatic islets. Such findings reinforce the view that these glibenclamide isosteres behave as potent insulin releasing agents [9].

The secretory effects of BM 208 and BM 225 were accompanied by modifications in transmembrane cationic

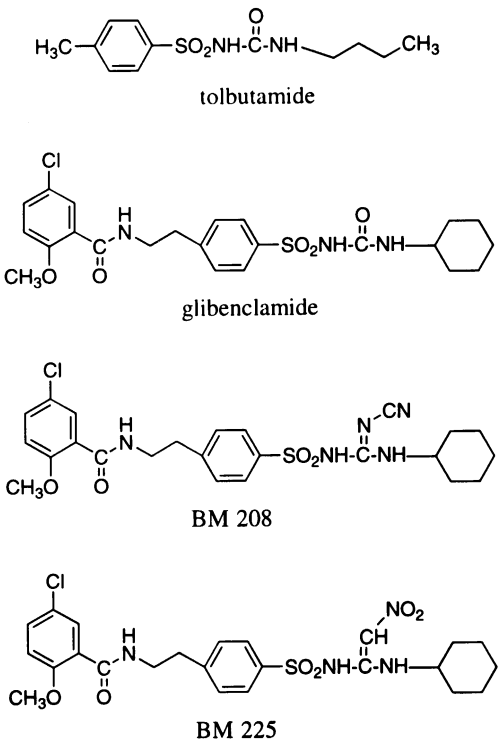

Fig. 6. Chemical structure of tolbutamide, glibenclamide, BM 208 and BM 225.

movements. Indeed, in the presence of 8.3 mM D-glucose in the experimental medium, both compounds increased ^{45}Ca outflow from prelabeled and perifused rat pancreatic islets. In islets exposed to Ca^{2+}-free media, however, the addition of BM 208 or BM 225 did not affect the ^{45}Ca outflow rate. This pattern is reminiscent of the previously reported effects of other hypoglycaemic sulfonylureas such as tolbutamide [12,16], gliclazide [17], glibenclamide [18,19] or glimepiride [18,19]. Taken as a whole, our radioisotopic data indirectly suggest that in the presence of 8.3 mM D-glucose in the perifusing medium, the stimulatory effect of BM 208 and BM 225 on ^{45}Ca outflow corresponds to a process of exchange between incoming non-radioactive Ca^{2+} and cellular $^{45}Ca^{2+}$.

The lack of effect of BM 208 and BM 225 on ^{45}Ca outflow from islets exposed to Ca^{2+}-free media further indicates that these glibenclamide isosteres do not promote an intracellular Ca^{2+} redistribution [16,20].

Moreover, experiments conducted on single rat pancreatic islet cells loaded with the fluorescent Ca^{2+} indicator fura-2 and incubated in the presence of 8.3 mM D-glucose revealed the capacity of BM 208 and BM 225 to raise the $[Ca^{2+}]_i$.

Thus, the combined data presented here indicated that in the presence of an extracellular glucose concentration close to the plasma concentration in fed rats, the insulinotropic action of BM 208 and BM 225 results from a facilitation of Ca^{2+} influx into the pancreatic B-cell.

Our findings also showed that the capacity of BM 225 to raise ^{45}Ca outflow, $[Ca^{2+}]_i$ and insulin release was more pronounced than that of BM 208. Moreover, in the presence of 8.3 mM D-glucose, the enhancing effect of BM 225 on both ^{45}Ca outflow and insulin release persisted when the administration of the compound was interrupted. Such an absence of reversibility of the insulinotropic response has been observed previously, under identical experimental conditions, with glibenclamide and glimepiride [18,19]. By contrast, and whatever the concentration tested, the effects of BM 208 on ^{45}Ca outflow and insulin output were reversible. In the presence of 8.3 mM glucose in the basal medium, the cationic and secretory responses to tolbutamide, gliclazide and glipizide have also been reported to be reversed upon their removal from the perifusate [12,17,21]. The higher lipophilicity of nitroethylene derivatives [8,22], as well as the more restricted molecular conformation imposed by the cyanoguanidine function [23], might explain that the effects of BM 225 (a nitroethylene derivative) were more pronounced and sustained than those of BM 208 (a cyanoguanidine derivative).

The observation of a reversibility of the BM 208 responses made it attractive to consider the effect of iterative administrations of BM 208 on ^{45}Ca outflow and insulin release from islets perifused at a physiological concentration of D-glucose (8.3 mM); such as that found in the plasma of fed rats. The successive addition of BM 208 provoked repeated increments in ^{45}Ca outflow and insulin release. These stimulatory effects displayed comparable magnitude at the occasion of each stimulation and were rapidly reversible upon withdrawal of the compound. Such findings provide direct support for the view that BM 208 could be considered as a suitable agent to cause transient stimulation of insulin release without inducing tachyphylaxis.

Lastly, the putative hypoglycaemic activities of BM 208 and BM 225 were assessed by measuring blood glucose concentration in Wistar rats. After intraperitoneal administration to fed rats, both compounds lowered basal plasma glucose concentration in a dose-dependent manner. The results further indicated that the potency of BM 225 was higher and its onset of action was faster than that of BM 208. Taken together, these *in vivo* and *in vitro* data suggest that the hypoglycaemic effects of both compounds result from their insulin-releasing action.

In conclusion, the present findings demonstrate that the administration of two newly synthesized isosteres of glibenclamide to rat pancreatic islets perifused at a physiological concentration of D-glucose (8.3 mM) elicits exocytotic releases of insulin. The secretory capacity of BM 208 and BM 225 was related to a facilitation of Ca^{2+} inflow into the pancreatic B-cell.

The *in vitro* experiments also reveal that the potency and duration of action of the two compounds were different. Indeed, the nitroethylene derivative (BM 225) exerted a strong and long lasting secretory effect whilst the release of insulin evoked by the cyanoguanidine derivative (BM 208) was weaker and reversible. The latter compound was able

to provoke, at the occasion of iterative administrations, repeated secretory responses without evidence of tachyphylaxis. This feature needs to be stressed because, when considering the administration of hypoglycaemic sulfonylureas to diabetic patients, a short duration of action has been reported to reduce the risk of hypoglycaemia and the risk of desensitization of the B cell response [1,24,25].

The two novel insulin releasing agents described here also exerted hypoglycaemic effects in normal rodents. Their activity profile was again dissimilar with a higher potency and a faster onset of action for BM 225.

Thus, taken as a whole, our results clearly indicate that appropriate structural modification of hypoglycaemic sulfonylureas can generate original bioisosteres with different features and activity profiles. From a clinical point of view, such an approach opens the prospect for the design of new insulinotropic agents in the treatment of type II diabetes mellitus.

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