

Direct measurements of increased free cytoplasmic Ca^{2+} in mouse pancreatic β -cells following stimulation by hypoglycemic sulfonylureas

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The effects of the hypoglycemic sulfonylureas tolbutamide and glibenclamide on free cytoplasmic Ca^{2+} , $[\text{Ca}^{2+}]_i$, were compared with that of a depolarizing concentration of K^+ in dispersed and cultured pancreatic β -cells from *ob/ob* mice. $[\text{Ca}^{2+}]_i$ was measured with the fluorescent Ca^{2+} -indicator quin2. The basal level corresponded to 150 nM and increased to 600 nM after exposure to 30.9 mM K^+ . The corresponding levels after stimulation with 1 μM glibenclamide and 100 μM tolbutamide were 390 and 270 nM respectively. K^+ depolarization increased $[\text{Ca}^{2+}]_i$ more rapidly than either of the sulfonylureas. It is suggested that the increased $[\text{Ca}^{2+}]_i$ obtained after stimulation by sulfonylureas is due to depolarization of the β -cells with subsequent entry of Ca^{2+} through voltage-dependent channels.

<i>Pancreatic β-cell</i>	<i>Cytoplasmic Ca^{2+}</i>	<i>Quin2</i>	<i>Hypoglycemic sulfonylurea</i>
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1. INTRODUCTION

It is well established that the insulin release evoked by hypoglycemic sulfonylureas is dependent upon adequate concentrations of extracellular Ca^{2+} [1]. Furthermore, it has been demonstrated that these drugs promote Ca^{2+} influx into the pancreatic β -cells, an effect probably due to depolarization with subsequent opening of voltage-dependent Ca^{2+} channels [2–5]. The action of sulfonylureas on ^{45}Ca efflux from preloaded islets differed from that of glucose in lacking the inhibitory component which is unmasked by lowering the extracellular Ca^{2+} concentration [6]. In a Ca^{2+} -deficient medium the drugs neither inhibited nor stimulated ^{45}Ca efflux [6]. These latter observations lend support to the view that the increased cytoplasmic Ca^{2+} concentration, necessary for activation of the insulin secretory machinery after sulfonylurea exposure, is promoted by an enhanced membrane permeability to the ion without much involvement of intracellular Ca^{2+} stores [6].

In this communication advantage was taken of the fluorescent intracellular Ca^{2+} -indicator quin2 for direct measurements of free cytoplasmic Ca^{2+} , $[\text{Ca}^{2+}]_i$, in normal pancreatic β -cells derived from obese-hyperglycemic mice [7–12]. A comparison was made between the $[\text{Ca}^{2+}]_i$ values obtained after stimulation by either the hypoglycemic sulfonylureas tolbutamide and glibenclamide or depolarizing concentrations of K^+ .

2. MATERIALS AND METHODS

All reagents were of analytical grade and redistilled deionized water was used. Quin2/AM and the ionophore A23187 were obtained from Calbiochem^R (Behring Diagnostics, La Jolla, CA). Dimethyl sulfoxide (DMSO) was from Sigma (St. Louis, MO). Boehringer Mannheim GmbH (Mannheim, FRG) supplied collagenase (EC 3.4.24.3). Glibenclamide and tolbutamide were generous gifts from Hoechst AG (Frankfurt am Main, FRG). Tissue culture medium RPMI 1640,

bovine fetal serum, penicillin and streptomycin were from Gibco, Scotland and gentamicin from Essex Chemie AG (Lucerne, Switzerland).

The experimental medium was a Hepes buffer, balanced in cations with Cl^- as the sole anion [13]. The Ca^{2+} concentration was 1.2 mM and the pH adjusted to 7.4 with NaOH. To avoid dilution of the cell suspension all additions were made from concentrated stock solutions.

The cell aggregates were prepared as described by Rorsman et al. [12]. Adult obese-hyperglycemic mice (*ob/ob*) of both sexes were taken from a local non-inbred colony [14]. The mice were starved overnight and the pancreases were removed immediately after killing. A collagenase digestion technique was used to isolate 500–1000 islets. The islets were disrupted gently to obtain a cell suspension where the cells remained in pauci-cellular aggregates [15]. To remove damaged cells the suspension was centrifuged through 10 ml of the basal medium supplemented with 4% albumin. The pelleted cells were then resuspended in 25 ml of RPMI 1640 tissue culture medium containing 10% fetal bovine serum, 100 IU/ml penicillin, 200 $\mu\text{g}/\text{ml}$ gentamicin and 100 $\mu\text{l}/\text{ml}$ streptomycin. This suspension was kept in the tissue culture medium for 24–30 h at 37°C and gassed with air containing 5% CO_2 . To prevent attachment of the cells to the flask it was gently shaken. In the resulting preparation most of the cells were in small aggregates. The preparation was routinely inspected under phase contrast before loading with quin2/AM.

Quin2/AM was added from a 50 mM stock solution in DMSO to the tissue culture medium (cell density $2\text{--}4 \times 10^5$ cells/ml), the final concentrations of quin2/AM and DMSO in the medium being 50 μM and 0.1%, respectively. After 60 min of loading, the cells were centrifuged for 2 min at $50 \times g$ and washed twice in 10 ml of the incubation medium. The cells were then resuspended in 1.25 ml of the basal incubation medium and transferred to the cuvette. Fluorescence was measured in an Aminco-Bowman spectrofluorometer with excitation and emission wavelengths set at 339 and 492 nm. The instrument was slightly modified to permit continuous magnetic stirring. All measurements were performed at 37°C. The procedure for calibrating the fluorescence signal was the same as that previously described by Rorsman and Abra-

hamsson [15]. To achieve F_{max} 10 mM Ca^{2+} and 20 mM Tris were added in addition to the 150 nM of the ionophore A23187. The autofluorescence was assessed by adding 1–2 mM Mn^{2+} to quench quin2 fluorescence once the F_{max} had been obtained. It was ascertained from separate experiments that the background fluorescence was not much altered by the various additions. The fluorescence signal was also corrected for the presence of extracellular quin2; the contribution of which was interpolated from measurements at the beginning and end of each experiment. The K_d of the Ca^{2+} -quin complex was assumed to be 115 nM [8]. Account was also taken of photobleaching of the quin2 fluorescence, which in separate experiments was found to be less than 10% over 30 min. Intracellular concentrations of quin2 were calculated using the F_{max} value as outlined above. The F_{max} value was compared with a calibration curve obtained by adding the free acid to the incubation medium. The intracellular concentration of quin2 was estimated assuming the β -cells to be spheres with a diameter of 13 μm [16]. Since 1 mg of protein corresponds to 5×10^6 cells [17] the intracellular volume can be calculated to be 5.5 $\mu\text{l}/\text{mg}$ protein. The resulting intracellular concentration of quin2 is probably an underestimate of the actual cytoplasmic concentration, since quin2 is not trapped within cellular organelles [8].

3. RESULTS AND DISCUSSION

It has been demonstrated that only sulfonylureas with insulin releasing activity promote the entry of Ca^{2+} into pancreatic β -cells [18]. Both electrophysiological data, revealing depolarization with subsequent appearance of action-potential-like spikes, and the rapid initiation of ^{45}Ca uptake, decaying with time, support the concept that Ca^{2+} influx is mediated through voltage-dependent channels [19–22]. Sulfonylurea-stimulated insulin release is therefore believed to be initiated by an increase in $[\text{Ca}^{2+}]_i$ although no direct experimental data have been available. In the present study advantage was taken of the fluorescent Ca^{2+} -indicator quin2 [7] for measuring directly changes in $[\text{Ca}^{2+}]_i$ following exposure to sulfonylureas at concentrations known to stimulate insulin release.

As demonstrated in figs 1 and 2 $[\text{Ca}^{2+}]_i$ increased promptly from 147 ± 5 nM ($n=7$) to

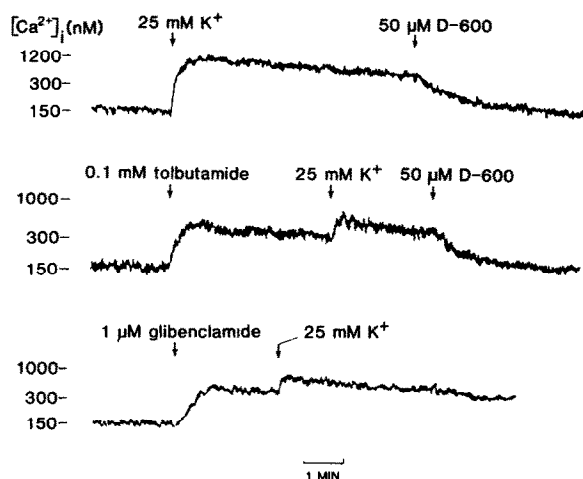


Fig.1. Effects of K^+ , D-600, tolbutamide and glibenclamide on intracellular free cytoplasmic Ca^{2+} . Fluorescence traces obtained from dispersed and cultured pancreatic β -cells loaded with quin2. After loading the β -cells (2–3 mg protein) were suspended in 1.25 ml of the basal incubation medium. Approximate values of $[Ca^{2+}]_i$ as well as the effects of K^+ , D-600, tolbutamide and glibenclamide are indicated.

272 ± 52 nM ($n=4$) and 386 ± 41 nM ($n=3$) after the addition of $100 \mu M$ tolbutamide and $1 \mu M$ glibenclamide, respectively. In this context it should be noted that neither of the additions changed the pH of the media. It is evident that glibenclamide is more potent than tolbutamide, although not as effective as 30.9 mM potassium in raising $[Ca^{2+}]_i$. A comparison of $[Ca^{2+}]_i$ values clearly demonstrated that the subsequent increase after exposure to the sulfonylureas was somewhat delayed compared to that obtained after stimulation by high K^+ . When discussing differences in potency between tolbutamide and glibenclamide it should be remembered that the latter drug is exceptional among hypoglycemic sulfonylureas in accumulating progressively in the β -cells [23]. The discrepancies in $[Ca^{2+}]_i$ values were not due to differences in intracellular quin2 content, the latter corresponding to 2.4 ± 0.4 mM ($n=6$), 2.5 ± 0.9 mM ($n=4$) and 3.2 ± 0.3 mM ($n=3$) for K^+ , tolbutamide and glibenclamide, respectively.

Although it has been suggested that the effect of hypoglycemic sulfonylureas on the β -cell handling of Ca^{2+} is mediated by an ionophoretic action of the compounds [24–26] convincing arguments

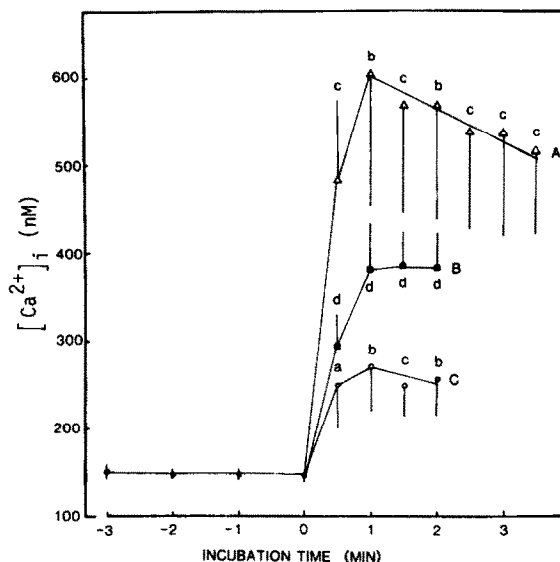


Fig.2. Effects of K^+ , glibenclamide and tolbutamide on intracellular free cytoplasmic Ca^{2+} . The $[Ca^{2+}]_i$ was calculated from experiments like that shown in fig.1. At 0 min, K^+ (A), glibenclamide (B) or tolbutamide (C) was added to the medium. Mean values $\pm$ SE for 3–7 experiments. Significances for the effects of the various additives were calculated using Student's t -test comparing the observed value with that measured immediately before the respective additions. ^a $P < 0.025$, ^b $P < 0.010$, ^c $P < 0.005$, ^d $P < 0.001$.

against this hypothesis have been presented [6]. This study revealed a similar stimulatory pattern of tolbutamide, glibenclamide and high K^+ on normal pancreatic β -cells resulting in increased values of $[Ca^{2+}]_i$. Addition of 25 mM K^+ to the sulfonylurea stimulated cells (fig.1) did not increase $[Ca^{2+}]_i$ in excess of that obtained with K^+ depolarization. Furthermore exposure to D-600 immediately reduced $[Ca^{2+}]_i$ to non-stimulatory values independently of whether the β -cells had been stimulated by high K^+ or hypoglycemic sulfonylureas (fig.1). These observations support the notion that hypoglycemic sulfonylureas depolarize the β -cells and favour the entry of Ca^{2+} through voltage-dependent channels rather than acting as Ca^{2+} ionophores.

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