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Sulphonylureas do not increase insulin secretion by a mechanism other than a rise in cytoplasmic Ca^{2+} in pancreatic B-cells

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Abstract

The following sequence of events is thought to underlie the stimulation of insulin release by hypoglycaemic sulphonylureas. Interaction of the drugs with a high-affinity binding site (sulphonylurea receptor) in the B-cell membrane leads to closure of ATP-sensitive K^+ channels, depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx and rise in cytoplasmic $[Ca^{2+}]_i$. Recent experiments using permeabilized islet cells or measuring changes in B-cell membrane capacitance have suggested that sulphonylureas can increase insulin release by a mechanism independent of a change in $[Ca^{2+}]_i$. This provocative hypothesis was tested here with intact mouse islets. When B-cells were strongly depolarized by 60 mM K^+, $[Ca^{2+}]_i$ was increased and insulin secretion stimulated. Under these conditions, tolbutamide did not further increase $[Ca^{2+}]_i$ or insulin release, whether it was applied before or after high K^+, and whether the concentration of glucose was 3 or 15 mM. This contrasts with the ability of forskolin and phorbol 12-myristate 13-acetate (PMA) to increase release in the presence of high K^+. Tolbutamide also failed to increase insulin release from islets depolarized with barium (substituted for extracellular Ca^{2+}) or with arginine in the presence of high glucose. Glibenclamide and its non-sulphonylurea moiety meglitinide were also without effect on insulin release from already depolarized B-cells. In the absence of extracellular Ca^{2+}, acetylcholine induced monophasic peaks of $[Ca^{2+}]_i$ and insulin secretion which were both unaffected by tolbutamide. Insulin release from permeabilized islet cells was stimulated by raising free Ca^{2+} (between 0.1 and 23 μM). This effect was not affected by tolbutamide and inconsistently increased by glibenclamide. In conclusion, the present study does not support the proposal that hypoglycaemic sulphonylureas can increase insulin release even when they do not also raise $[Ca^{2+}]_i$ in B-cells.

Keywords: Sulphonylurea; Tolbutamide; Glibenclamide; Pancreatic B-cell; Insulin release; Stimulus-secretion coupling; Ca^{2+}, cytoplasmic; K^+ channel, ATP-sensitive; Islet, mouse

1. Introduction

Hypoglycaemic sulphonylureas have been used for 40 years and remain the only clinically useful drugs to stimulate insulin release in non-insulin-dependent diabetic patients (Henquin, 1992).

An essential step in the complex chain of events leading to the stimulation of insulin release by glucose is the closure of ATP-sensitive K^+ channels (K^+-ATP channels) in the plasma membrane of pancreatic B-cells. This closure is achieved by the rise in ATP/ADP ratio (and perhaps other signals) that occurs upon acceleration of glucose metabolism. Its consequence is a depolarization of the membrane, with subsequent opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx and rise in cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ (for review, Dunne and Petersen, 1991; Misler et al., 1992; Henquin, 1994).

Sulphonylureas mimic some of the effects of glucose. They depolarize the B-cell membrane (Henquin and Meissner, 1982) by closing K^+-ATP channels (Sturgess et al., 1985; Trube et al., 1986), thereby increasing Ca^{2+} influx and leading to a rise in $[Ca^{2+}]_i$ (Abrahamsson et al., 1985), that triggers insulin release. A major difference between both secretagogues is that the closure of K^+-ATP channels by sulphonylureas does not result from changes in B-cell metabolism but from a direct interaction with high affinity binding sites, considered as sulphonylurea receptors (Nelson et al., 1992; Ashcroft and Ashcroft, 1992). Whether this receptor is an integral part of the K^+-ATP channels or a distinct regulatory protein has been much disputed. The observation that one insulin-secreting cell line (CRI-D11 cells) possesses a normal number of K^+-ATP channels and only few sulphonylurea receptors was consistent with both structures being distinct (Khan et al., 1993). Although this

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view is supported by the lack of effect of sulphonylureas on cloned K^+-ATP channels expressed in oocytes (Ashford et al., 1994), and by the fact that the cloned sulphonylurea receptor does not form a channel (Aguilar-Bryan et al., 1995), the available evidence is not yet completely decisive.

It has recently been suggested that binding of sulphonylureas to their receptor might increase insulin release by mechanisms other than the closure of K^+-ATP channels and the rise in $[Ca^{2+}]_i$ in B-cells. This provocative hypothesis was based on two sets of observations. First, tolbutamide and glibenclamide were found to increase insulin release from permeabilized RINm5F or islet cells, in which the membrane potential is dissipated and $[Ca^{2+}]_i$ clamped (Flatt et al., 1994). Second, in voltage-clamped single B-cells, tolbutamide increased exocytosis, estimated by measurements of membrane capacitance changes, without affecting Ca^{2+} currents (Ämmälä et al., 1994). The finding that the majority of sulphonylurea receptors are localized to intracellular membranes in insulinoma cells (Ozanne et al., 1995) might also suggest that sulphonylureas have multiple sites of action in B-cells.

The evidence that sulphonylureas might increase insulin release at fixed $[Ca^{2+}]_i$ has been obtained under very peculiar experimental conditions dictated by the technical approaches that were used. It is, therefore, essential to test this hypothesis with intact cells in which $[Ca^{2+}]_i$ cannot be affected by sulphonylureas, but from which insulin release can still be stimulated. This was achieved here, with intact mouse islets perifused or incubated under depolarizing conditions or in the absence of extracellular Ca^{2+}.

2. Materials and methods

2.1. Preparation and solutions

All experiments, except those using permeabilized cells (see below), were performed with intact islets isolated by collagenase digestion of the pancreas of fed female NMRI mice. Except where otherwise stated, the medium used was a bicarbonate-buffered solution containing (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and $NaHCO_3$ 24. It was gassed with O_2/CO_2 (94/6) to maintain pH 7.4 and was supplemented with 1 mg ml^{-1} bovine serum albumin (Boehringer, Mannheim, Germany). Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$, and the concentration of residual Ca^{2+} was less than 10 μM. When the concentration of KCl was increased to 60 mM that of NaCl was decreased to 65 mM to keep the osmolarity of the medium unchanged.

2.2. Measurements of insulin release from intact islets

After isolation, the islets were preincubated for 1 h in a medium containing 15 mM glucose, a concentration that produces half-maximum stimulation of mouse B-cells. In one type of experiments, they were then incubated for 60 min, in batches of three, in 1 ml of medium containing appropriate concentrations of test subtances. At the end of the incubation, a portion of the medium was withdrawn and diluted before insulin assay. In another type of experiments, preincubated islets were placed, in batches of 20 or 35 (depending on the protocol) in parallel perifusion chambers. Effluent fractions were collected at 1 or 2 min intervals for insulin assay. In one series, the islets were cultured overnight in RPMI medium containing 10 mM glucose before being used in perifusion experiments. Insulin was measured by a double-antibody radioimmunoassay with rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark).

2.3. Measurements of insulin release from permeabilized islet cells

After isolation, the islets were rinsed with a medium containing 10 mM glucose, no $CaCl_2$ and 100 μM EGTA. They were then incubated at 37°C for 4–5 min in the same medium supplemented with 0.1 mg trypsin ml^{-1}, with gentle pipetting through a siliconized glass pipette, until the islets disappeared. One minute later, cold RPMI with 10% fetal calf serum was added to stop trypsin digestion, cells were centrifuged 5 min at $1000 \times g$ and the supernatant was discarded. After a second wash with cold RPMI, the cells were resuspended in 4 ml RPMI and counted. They were then preincubated for 1 h at 37°C, in an atmosphere of 5% CO_2 in air. An average of 3000–4000 cells per islet was obtained by this method. Islet cell viability was higher than 90%, as revealed by the trypan blue dye uptake test.

Staphylococcus aureus α-toxin was purified as described (Palmer et al., 1993) and reconstituted at a concentration of 3.6 mg ml^{-1} in 10 mM phosphate-buffered saline, pH 7.0, containing 1 mg ml^{-1} bovine serum albumin. The haemolytic activity of the toxin was determined as recently described (Jonas et al., 1994a). To preserve toxin activity, aliquots were kept at −70°C until use.

After preincubation, islet cells were washed twice with a Ca^{2+}-free medium containing 400 μM EGTA. They were then resuspended at a concentration of 2×10^6 cells ml^{-1} in a buffer containing (mM): Hepes 20, K glutamate 140, NaCl 5, $MgSO_4$ 7, Na_2ATP 5 and EGTA 0.2. The pH was 7.0 and free Ca^{2+} approximately 0.1 μM. This cell suspension was mixed 1 : 1 with the same buffer supplemented with α-toxin and incubated for 10 min at 37°C at a final concentration of 2000 Haemolytic Units 10^6 $cells^{-1}$ ml^{-1}. After centrifugation (5 min at $1000 \times g$, 4°C), the supernatant was discarded and the cells were resuspended in buffer (10^5 cells ml^{-1}). Cell permeabilization was assessed by eosin and trypan blue uptake tests. Using this protocol, more than 90% of cells were permeable to trypan blue (molecular weight = 950) and 100% of the cells were permeable to eosin G (molecular weight = 624).

Permeabilized cells were then incubated (10 min at 4°C followed by 15 min at 37°C) at a concentration of 10^4 cells ml^{-1}. The buffer contained 10.2 mM EGTA and appropriate amounts of $CaCl_2$ to achieve free Ca^{2+} concentrations of 0.1 μM, 2.6 μM and 23 μM as calculated with a computer program developed by Föhr et al. (1993). The test substances were present only during this incubation or also during the preincubation and permeabilization steps (see legend to Fig. 7). At the end of the incubation, the tubes were centrifuged for 2 min at 10000 × g (4°C), and an aliquot of the supernatant was adequately diluted for insulin assay. In each experiment, cellular insulin content was determined on an aliquot of permeabilized cells used for incubation.

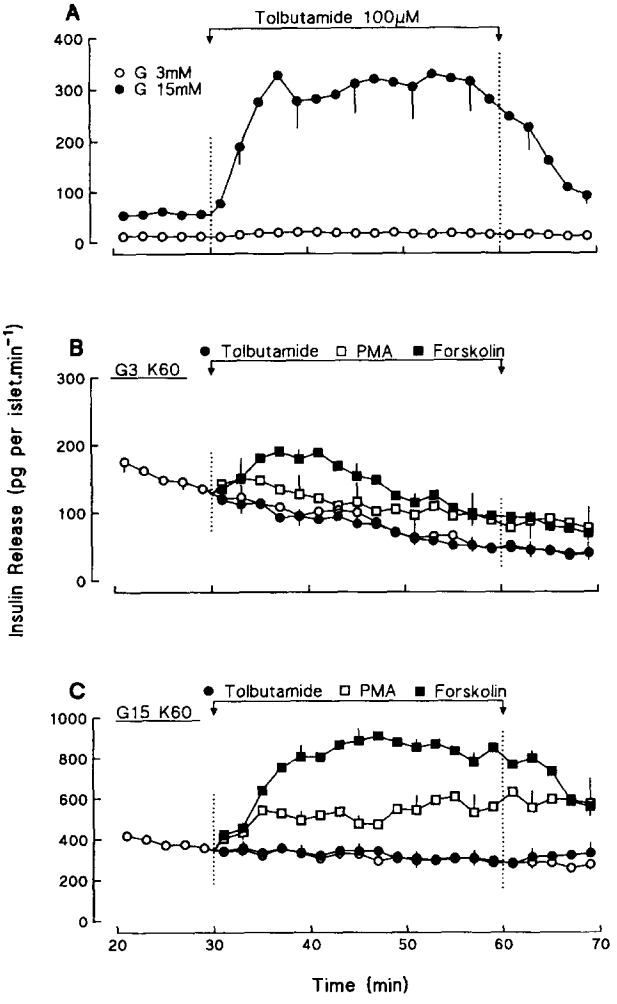

Fig. 1. Effects of tolbutamide, a phorbol ester (PMA) and forskolin on insulin release from perifused mouse islets. Groups of 20 islets were perifused throughout with a medium containing: (A) 4.8 mM KCl and either 3 or 15 mM glucose (G), (B) 60 mM KCl and 3 mM glucose, or (C) 60 mM KCl and 15 mM glucose. Test substances were added between 30 and 60 min: (A) tolbutamide was added to both groups of islets, (B and C) 100 μM tolbutamide, 10 nM PMA or 1 μM forskolin was added to groups of islets indicated by the corresponding symbol, whereas control islets not challenged with a test substance are shown by (○). Values are means ± S.E.M. for four to six experiments.

2.4. Measurements of cytosolic $[Ca^{2+}]_i$

Isolated islets were cultured overnight in RPMI medium containing 10 mM glucose (Gilon and Henquin, 1992). They were then loaded with fura-2 during a preincubation of 40 min in the presence of 10 mM glucose. After washing, they were transferred to a Ca^{2+}-measuring system in which the perifused tissue was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science, Turnbridge Wells, UK). The images were analyzed by the system MagiCal (Applied Imaging, Sunderland, UK). The technique has been described in detail previously (Gilon and Henquin, 1992).

2.5. Drugs

Tolbutamide, glibenclamide and meglitinide were obtained from Hoechst AG (Frankfurt/Main, Germany), phentolamine from Ciba-Geigy (Basel, Switzerland), the phorbol ester PMA (phorbol 12-myristate 13-acetate), quinine and acetylcholine from Sigma (St Louis, MO, USA), and forskolin from Calbiochem-Behring (San Diego, CA, USA). RPMI medium was from Gibco (Paisley, Scotland, UK) and the *Staphylococcus aureus* α-toxin was kindly provided by Dr. M. Palmer (Mainz, Germany).

2.6. Statistical analysis

Except for certain measurements of cytosolic $[Ca^{2+}]_i$, results are presented as means ± S.E.M. for the indicated number of experiments or batches of islets (from the indicated number of different preparations). The statistical significance of differences between means was assessed by an unpaired t-test, or by analysis of variance followed by a Newman-Keuls test when more than two groups were compared. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Control effects of tolbutamide

When mouse islets were perifused with a control medium containing a non-stimulatory concentration of glucose (3 mM), the rate of insulin release was low, but was slightly increased by the addition of 100 μM tolbutamide. This small change from 12 ± 2 pg $islet^{-1}$ min^{-1} to 20 ± 2 pg $islet^{-1}$ min^{-1} is hardly visible in Fig. 1A. On the other hand, when insulin release was stimulated by 15 mM glucose, tolbutamide triggered a rapid, large and reversible increase in insulin release (Fig. 1A). We have previously reported that tolbutamide causes a large, sustained rise of $[Ca^{2+}]_i$ in islet cells in the presence of either low or high glucose (Gilon and Henquin, 1992).

3.2. Effects of tolbutamide in depolarized B-cells

When the islets were perifused with a medium containing 60 mM K^+, insulin release was stimulated. This stimulation was stronger and more sustained in the presence of 15 mM glucose (Fig. 1C) than 3 mM glucose (Fig. 1B). Addition of 100 μM tolbutamide to the high K^+ medium was without effect on insulin release at either glucose concentration. This contrasts with the increases in release that followed the activation of protein kinase C with 10 nM PMA or of adenylate cyclase (hence of protein kinase A) with 1 μM forskolin (Fig. 1B,C).

During perifusion with a control medium containing 4.8 mM K^+, $[Ca^{2+}]_i$ in islet cells was low and fairly stable in the presence of 3 mM glucose, but was higher and oscillating in the presence of 15 mM glucose (Fig. 2). Raising K^+ to 60 mM triggered a rapid increase in $[Ca^{2+}]_i$ with a large initial peak and a secondary stabilization in plateau. The subsequent addition of 100 μM tolbutamide was followed by a minor but consistent decrease in $[Ca^{2+}]_i$ from 194 ± 9 nM to 181 ± 9 nM in the presence of 3 mM glucose (Fig. 2A). It was without effect in the presence of 15 mM glucose (Fig. 2B): average $[Ca^{2+}]_i$ amounted to 200 ± 11 nM before, and 206 ± 11 nM after addition of tolbutamide.

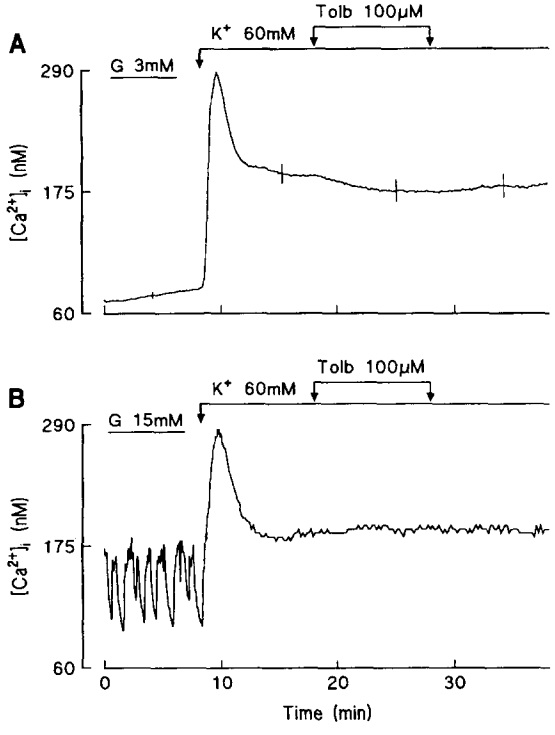

Fig. 2. Effects of tolbutamide on the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in islet cells perifused with a medium containing 60 mM K^+. After loading with fura-2, islets were perifused with a medium containing 4.8 mM KCl and either 3 mM (A) or 15 mM (B) glucose (G). The concentration of KCl was then raised to 60 mM and 100 μM tolbutamide added as indicated. Panel A shows the means ± S.E.M. for 15 islets. Panel B is representative of the response obtained in 15 islets; the recordings were not averaged because of the oscillations of $[Ca^{2+}]_i$.

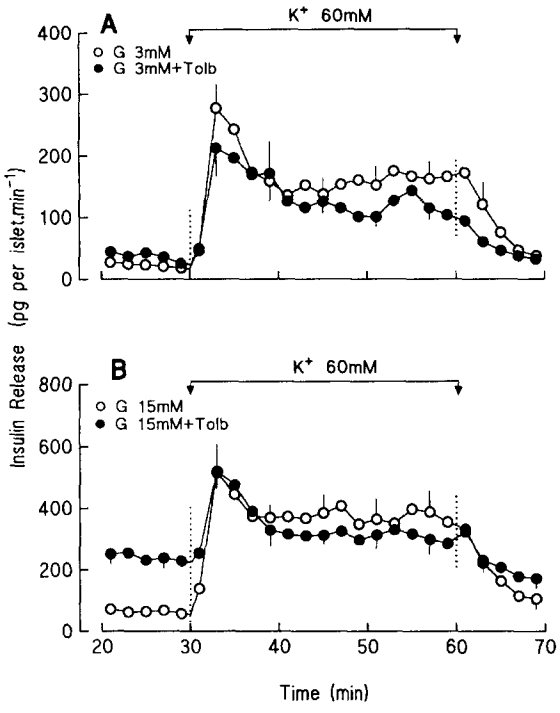

Fig. 3. Effects of high K^+ on insulin release from perifused islets. Groups of 20 islets were perifused with a medium containing 3 mM (A) or 15 mM (B) glucose (G) without (○) or with 100 μM tolbutamide throughout. Between 30 and 60 min, the concentration of KCl in the medium was raised from 4.8 to 60 mM. Values are means ± S.E.M. for five experiments.

In another series of experiments, the islets were treated with tolbutamide in a control medium before stimulation with high K^+ (Fig. 3). Tolbutamide produced its usual glucose-dependent increase in insulin release in the presence of 4.8 mM K^+, but did not potentiate the response to 60 mM K^+.

Sustained depolarization of the B-cell membrane can also be produced by substituting $BaCl_2$ for $CaCl_2$ (Ribalet and Beigelman, 1981) or by addition of arginine to a medium containing a stimulatory concentration of glucose (Henquin and Meissner, 1981). Control insulin release induced by 10 mM glucose was increased ~ 8-fold by 2.5 mM $BaCl_2$, while the effect of 15 mM glucose was increased 4.5-fold by 20 mM arginine (Fig. 4). The effectiveness of these combinations was not affected by 100 μM tolbutamide but was augmented by PMA or forskolin.

3.3. Effects of tolbutamide on insulin release in the absence of extracellular Ca^{2+}

When the islets were perifused with a medium without $CaCl_2$, $[Ca^{2+}]_i$ in islet cells was low and stable despite the presence of 15 mM glucose, and was not different in the presence or absence of tolbutamide (Fig. 5). The rate of insulin release was also low and similar with and without tolbutamide. Addition of acetylcholine to the Ca^{2+}-free medium triggered a large, transient, rise of $[Ca^{2+}]_i$ and a

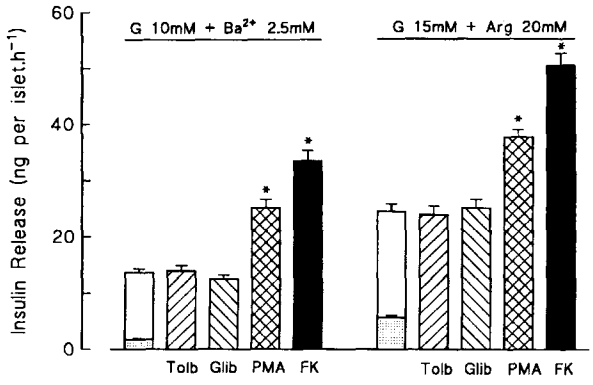

Fig. 4. Effects of tolbutamide (Tolb), glibenclamide (Glib), a phorbol ester (PMA) and forskolin (FK) on insulin release from incubated mouse islets. Batches of three islets were incubated for 60 min in 1 ml of medium containing 10 mM glucose (G) and 2.5 mM $BaCl_2$ instead of $CaCl_2$ (left-hand columns) or 15 mM glucose (G) and 20 mM arginine (right-hand columns). The shaded columns within the open columns show the release of insulin by control islets incubated in 10 or 15 mM glucose alone. The test substances were used at the concentrations of Tolb (100 μM), Glib (1 μM), PMA (25 nM), forskolin (1 μM). Values are means ± S.E.M. for 20–25 batches of islets from four (Ba^{2+}) or five (arginine) experiments. * $P < 0.01$ vs. controls.

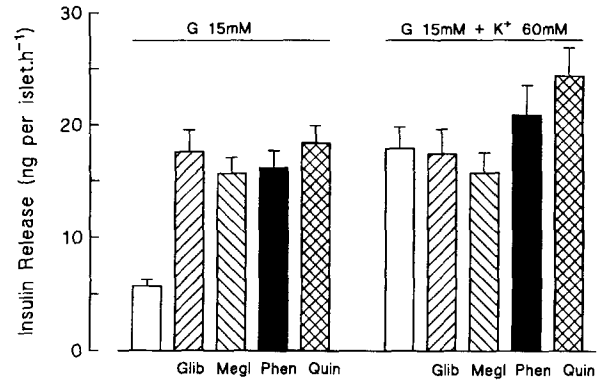

Fig. 6. Effects of various blockers of K^+-ATP channels on insulin release from mouse islets incubated in the presence of 15 mM glucose (G) and 4.8 mM K^+ (left-hand columns) or 15 mM G and 60 mM K^+ (right-hand columns). Batches of three islets were incubated in one of these media supplemented or not with 1 μM glibenclamide (Glib), 50 μM meglitinide (Megl), 100 μM phentolamine (Phen) or 50 μM quinine (Quin) as indicated. Values are means ± S.E.M. for 15 batches of islets from three separate experiments.

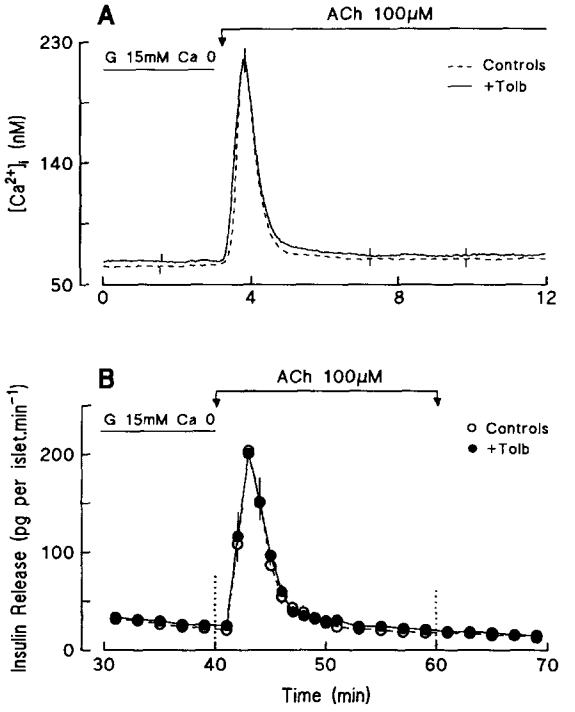

Fig. 5. Lack of influence of tolbutamide on the effects of acetylcholine (ACh 100 μM) on $[Ca^{2+}]_i$ and insulin secretion from mouse islets perifused with a Ca^{2+}-free medium. The changes in $[Ca^{2+}]_i$ were measured in individual islets loaded with fura-2, whereas insulin secretion experiments were carried out with groups of 35 islets per perifusion chamber. Overnight cultured islets were perifused with a medium containing 15 mM glucose and no $CaCl_2$, and supplemented or not with 100 μM tolbutamide (Tolb) as indicated. Values are means ± S.E.M. for 16 measurements of $[Ca^{2+}]_i$ and seven experiments of insulin release.

single peak of insulin release which were both unaffected by the presence of tolbutamide (Fig. 5).

3.4. Effects of agents other than tolbutamide

Glibenclamide, its non-sulphonylurea moiety, meglitinide, phentolamine and quinine block K^+-ATP channels like tolbutamide (Zünkler et al., 1988; Plant and Henquin, 1990; Bokvist et al., 1990). They all potentiated insulin release induced by 15 mM glucose in a control medium containing 4.8 mM K^+. On the other hand, these drugs did not significantly increase insulin release when B-cells were depolarized by high K^+ (although some trend was seen

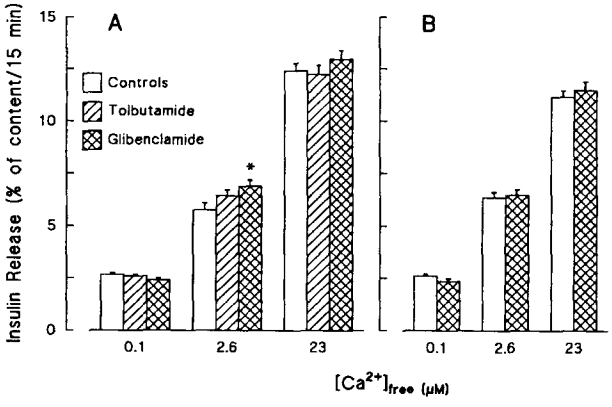

Fig. 7. Effects of tolbutamide and glibenclamide on insulin release from permeabilized islet cells. Dispersed islet cells were permeabilized with *Staphylococcus aureus* α-toxin and incubated for 15 min in the presence of the indicated concentration of free Ca^{2+}. In (A) tolbutamide (100 μM) and glibenclamide (1 μM) were added during the incubation only. In (B) glibenclamide was also present during the last 30 min of preincubation and during the permeabilization procedure. Values are means ± S.E.M. for 16–24 batches of cells from three to four separate experiments. * $P < 0.05$ vs. controls.

with phentolamine and quinine) (Fig. 6). Glibenclamide also failed to increase insulin release in the presence of barium or arginine (Fig. 4).

3.5. Effects of tolbutamide and glibenclamide in permeabilized islet cells

Micromolar concentrations of ionized Ca^{2+} induced a concentration-dependent increase in insulin release from islet cells permeabilized with the *Staphylococcus aureus* α-toxin (Fig. 7). This effect was totally abrogated by omission of ATP from the incubation medium (not shown). Tolbutamide was without effect on insulin release from these permeabilized cells. Glibenclamide marginally (by 15%) increased the effect of an intermediate concentration of Ca^{2+} when added acutely to the incubation medium (Fig. 7A). No effect of glibenclamide could be detected when the drug was already added to the cells before permeabilization (Fig. 7B).

4. Discussion

Experiments using permeabilized insulin-secreting cells (Flatt et al., 1994) or membrane capacitance measurements in single B-cells (Ämmälä et al., 1994) have led to the proposal that hypoglycaemic sulphonylureas might increase insulin release even when they do not raise $[Ca^{2+}]_i$ in B-cells. The present study, performed with intact mouse islets, does not support this hypothesis.

Previous observations that glucose and tolbutamide have more than additive effects on insulin release (Henquin, 1988; Panten et al., 1988) might suggest that both agents affect secretion by distinct mechanisms in addition to their common, classical, sequence of blockade of K^+-ATP channels, depolarization and stimulation of Ca^{2+} influx. This has indeed been established for glucose which also increases the sensitivity of the secretory process to $[Ca^{2+}]_i$ (Gembal et al., 1993). On the other hand, the ability of tolbutamide to increase insulin release in the presence of a maximally effective concentration of glucose (Panten et al., 1986; Henquin, 1988) can be explained by the fact that not all K^+-ATP channels are closed by very high glucose. Thus, even in the presence of 30–40 mM glucose, tolbutamide increases the frequency of Ca^{2+} action potentials (Henquin, 1988) and raises $[Ca^{2+}]_i$ in B-cells (P. Gilon, unpublished data).

Only very few experimental protocols are suitable to test whether the effects of sulphonylureas on insulin release can be dissociated from their effect on $[Ca^{2+}]_i$ in *intact cells*. As a first approach, $[Ca^{2+}]_i$ was steadily increased by depolarizing the B-cell membrane with 60 mM K^+. Under these conditions, insulin release was ~ 3-fold larger in the presence of 15 mM than in the presence of 3 mM glucose although average $[Ca^{2+}]_i$ was not different. This reflects the K^+-ATP channel-independent effect of glucose, which we have described and characterized recently (Gembal et al., 1993). Addition of tolbutamide to the high K^+ medium did not raise $[Ca^{2+}]_i$ further, as expected, but was also without effect on insulin release regardless of the concentration of glucose. This ineffectiveness on secretion cannot be attributed to an already maximal rate of insulin release because PMA and forskolin potentiated high K^+-induced insulin release. It cannot be ascribed to a particular sequence of stimulation since the insulin-releasing effect of high K^+ was not increased by tolbutamide pretreatment. It is not specifically linked to a mechanism of depolarization since tolbutamide also failed to potentiate insulin release from islets depolarized by the combination of 15 mM glucose and 20 mM arginine or by substitution of Ba^{2+} for extracellular Ca^{2+}. We attribute the lack of effect of tolbutamide on insulin release under these conditions to an inability to cause a sustained further rise in $[Ca^{2+}]_i$.

Besides tolbutamide, four blockers of K^+-ATP channels were tested in depolarized B-cells. Two of them, glibenclamide and meglitinide (the non-sulphonylurea moiety of glibenclamide), which also bind to the sulphonylurea receptor (Ashcroft and Ashcroft, 1992), did not increase insulin release under these conditions. On the other hand, a tendency to increase insulin release also in the presence of high K^+ was noted for quinine and phentolamine. Quinine does not bind to the sulphonylurea receptor (Schwanstecher et al., 1992) and it is unlikely that phentolamine binds to the receptor in B-cells for two reasons. First, it does not displace glibenclamide from its specific binding sites in brain and heart (Yamashita et al., 1995). Second, efaroxan, which has an imidazoline structure like phentolamine and shares the properties of the latter on K^+-ATP channels, does not bind to the receptor in insulin-secreting cells (Brown et al., 1993). Interestingly, phentolamine and other imidazolines increase glucose-stimulated insulin release more than do maximally effective concentrations of tolbutamide (Jonas et al., 1992, 1994b). Whether this reflects their ability to block voltage- and Ca^{2+}-dependent K^+ channels in addition to K^+-ATP channels (Plant and Henquin, 1990) or an additional, yet unidentified, action remains to be established.

Tolbutamide is known not to affect $[Ca^{2+}]_i$ in B-cells in the absence of extracellular Ca^{2+} (Nelson et al., 1992; Gilon and Henquin, 1992). This was confirmed by the present study, which further showed that the drug was without effect on the peak of $[Ca^{2+}]_i$ resulting from the mobilization of intracellular Ca^{2+} by acetylcholine. The concomitant release of insulin was also unaffected by tolbutamide, as should have occurred if the sulphonylurea were able to increase the efficacy of Ca^{2+} on the secretory system.

In contrast to Flatt et al. (1994), we did not observe any consistent effect of tolbutamide and glibenclamide on insulin release from permeabilized islet cells, which otherwise responded normally to a rise in free Ca^{2+} within the

micromolar range. Glibenclamide was ineffective even when it was applied before permeabilization. It is unlikely that these discrepancies can be explained by the use of different techniques of permeabilization because both the α-toxin and the electropermeabilization only produce small pores that do not allow loss of cytosolic proteins. Moreover, S.J. Persaud and P.M. Jones (personal communication) have also found 100 μM tolbutamide and 2 μM glibenclamide not to increase insulin release from electropermeabilized rat islets perifused with a medium containing 0.05 μM Ca^{2+}, conditions under which both sulphonylureas had a clear effect in the experiments of Flatt et al. (1994).

We acknowledge that the islets were submitted to unphysiological test conditions (high K^+ or Ca^{2+}-free medium). However, these were inescapable constraints to distinguish between effects of sulphonylureas mediated by changes in $[Ca^{2+}]_i$ and effects independent of these changes. During estimations of insulin release by recording membrane capacitance changes, $[Ca^{2+}]_i$ in B-cells increases much more than in any of our stimulatory conditions, but this did not prevent detection of a large effect of tolbutamide (Ämmälä et al., 1994). Our results, however, show that no biologically equivalent effect can be detected when insulin release is directly measured from the more physiological preparation of intact mouse islets.

Our study does not exclude the possibility that binding of sulphonylureas to their receptor produces effects other than closure of K^+-ATP channels. For instance, tolbutamide, in contrast to glucose, increases $[Na^+]_i$ in B-cells (Ali et al., 1989). It is still unclear whether this rise in $[Na^+]_i$ contributes to the insulin-releasing effect of sulphonylureas but, if this is the case, the effect of Na^+ is likely to be mediated by secondary changes in $[Ca^{2+}]_i$. This would be very different from the postulated sensitizing action (Flatt et al., 1994; Ämmälä et al., 1994), of which we have tested the existence in this study. The observation that many sulphonylurea receptors are intracellular has also been taken as support for additional effects of the drugs (Ozanne et al., 1995). It should, however, be borne in mind that the entry of sulphonylureas into B-cells is the exception (glibenclamide) rather than the rule (Gylfe et al., 1984; Panten et al., 1989).

In conclusion, the present study does not support the recent proposal that hypoglycaemic sulphonylureas can increase insulin release also when they do not also raise $[Ca^{2+}]_i$ in B-cells.

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References

Abrahamsson, H., P.O. Berggren and P. Rorsman, 1985, Direct measurements of increased free cytoplasmic Ca^{2+} in mouse pancreatic β-cells following stimulation by hypoglycemic sulfonylureas, FEBS Lett. 190, 21.

Aguilar-Bryan, L., C.G. Nichols, S.W. Wechsler, J.P. Clement, A.E. Boyd, G. Gonzalez, H. Herrera-Sosa, K. Nguy, J. Bryan and D.A. Nelson, 1995, Cloning of the β-cell high-affinity sulfonylurea receptor: a regulator of insulin secretion, Science 268, 423.

Ali, L., E. Grapengiesser, E. Gylfe, B. Hellman and P.-E. Lund, 1989, Free and bound sodium in pancreatic β-cells exposed to glucose and tolbutamide, Biochem. Biophys. Res. Commun. 164, 212.

Ämmälä, C., K. Bokvist, L. Eliasson, E. Rehnström and P. Rorsman, 1994, Sulphonylurea and exocytosis in mouse β-cells: a possible interaction with protein kinase C, Diabetologia 37 (Suppl. 1), A48 (abstract).

Ashcroft, S.J.H. and F.M. Ashcroft, 1992, The sulfonylurea receptor, Biochim. Biophys. Acta 1175, 45.

Ashford, M.L.J., C.T. Bond, T.A. Blair and J.P. Adelman, 1994, Cloning and functional expression of a rat heart K_{ATP} channel, Nature 370, 456.

Bokvist, K., P. Rorsman and P.A. Smith, 1990, Block of ATP-regulated and Ca^{2+}-activated K^+ channels in mouse pancreatic β-cells by external tetraethylammonium and quinine, J. Physiol. 423, 327.

Brown, C.A., S.L.F. Chan, M.R. Stillings, S.A. Smith and N.G. Morgan, 1993, Antagonism of the stimulatory effects of efaroxan and glibenclamide in rat pancreatic islets by the imidazoline, RX801080, Br. J. Pharmacol. 110, 1017.

Dunne, M.J. and O.H. Petersen, 1991, Potassium selective ion channels in insulin-secreting cells: physiology, pharmacology and their role in stimulus-secretion coupling, Biochim. Biophys. Acta 1071, 67.

Flatt, P.R., O. Shibier, J. Szecowka and P.O. Berggren, 1994, New perspectives on the actions of sulphonylureas and hyperglycaemic sulphonamides on the pancreatic β-cell, Diabete Metab. 20, 157.

Föhr, K.J., W. Warchol and M. Gratzl, 1993, Calculation and control of free divalent cations in solutions used for membrane fusion studies, Methods Enzymol. 221, 149.

Gembal, M., P. Detimary, P. Gilon, Z.Y. Gao and J.C. Henquin, 1993, Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse B-cells, J. Clin. Invest. 91, 871.

Gilon, P. and J.C. Henquin, 1992, Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell, J. Biol. Chem. 267, 20713.

Gylfe, E., B. Hellman, J. Sehlin and I.B. Täljedal, 1984, Interaction of sulfonylurea with the pancreatic B-cell, Experientia 40, 1126.

Henquin, J.C., 1988, ATP-sensitive K^+ channels may control glucose-induced electrical activity in pancreatic B-cells, Biochem. Biophys. Res. Commun. 156, 769.

Henquin, J.C., 1992, The fiftieth anniversary of hypoglycaemic sulphonamides. How did the mother compound work?, Diabetologia 35, 907.

Henquin, J.C., 1994, The cell biology of insulin secretion, in: The Joslin's Diabetes Mellitus, 13th edn., eds. G.C. Weir and C.R. Kahn (Lea and Febiger, Philadelphia) p. 56.

Henquin, J.C. and H.P. Meissner, 1981, Effects of amino acids on membrane potential and ^{86}Rb fluxes in pancreatic β-cells, Am. J. Physiol. 240, E245.

Henquin, J.C. and H.P. Meissner, 1982, Opposite effects of tolbutamide and diazoxide on $^{86}Rb^+$ fluxes and membrane potential in pancreatic B-cells, Biochem. Pharmacol. 31,1407.

Jonas, J.C., T.D. Plant and J.C. Henquin, 1992, Imidazoline antagonists of α_2-adrenoceptors increase insulin release in vitro by inhibiting ATP-sensitive K^+ channels in pancreatic β-cells, Br. J. Pharmacol. 107, 8.

Jonas, J.C., G. Li, M. Palmer, U. Weller and C.B. Wollheim, 1994a, Dynamics of Ca^{2+} and guanosine 5'-[γ-thio]triphosphate action on insulin secretion from α-toxin-permeabilized HIT-T15 cells, Biochem. J. 301, 523.

Jonas, J.C., T.D. Plant, I. Angel, S.Z. Langer and J.C. Henquin, 1994b, In vitro stimulation of insulin release by SL 84.0418, a new α_2-adrenoceptor antagonist, Eur. J. Pharmacol. 254, 27.

Khan, R.N., C.N. Hales, S.E. Ozanne, A.A. Adogu and M.L.J. Ashford, 1993, Dissociation of K_{ATP} channel and sulphonylurea receptor in the rat clonal insulin-secreting cell line, CRI-D11, Proc. R. Soc. London 253, 225.

Misler, S., D.W. Barnett, K.D. Gillis and D.M. Pressel, 1992, Electrophysiology of stimulus-secretion coupling in human β-cells, Diabetes 41, 1221.

Nelson, D.A., L. Aguilar-Bryan, H. Raef and A.E. Boyd, 1992, Molecular mechanisms of sulphonylurea action in the pancreatic B-cell, in: Nutrient Regulation of Insulin Secretion, ed. P.R. Flatt (Portland Press, London and Chapel Hill) p. 319.

Ozanne, S.E., P.C. Guest, J.C. Hutton and C.N. Hales, 1995, Intracellular localization and molecular heterogeneity of the sulphonylurea receptor in insulin-secreting cells, Diabetologia 38, 277.

Palmer, M., R. Jursch, U. Weller, A. Valeva, K. Hilgert, M. Kehoe and S. Bhakdi, 1993, *Staphylococcus aureus* α-toxin, production of functionnally intact, site-specifically modifiable protein by introduction of cystein at positions 69, 130 and 186, J. Biol. Chem. 268, 11959.

Panten, U., B.J. Zünkler, S. Scheit, K. Kirchhoff and S. Lenzen, 1986, Regulation of energy metabolism in pancreatic islets by glucose and tolbutamide, Diabetologia 29, 648.

Panten, U., M. Schwanstecher, A. Wallasch and S. Lenzen, 1988, Gucose both inhibits and stimulates insulin secretion from isolated pancreatic islets exposed to maximally effective concentrations of sulfonylureas, Naunyn-Schmied. Arch. Pharmacol. 338, 459.

Panten, U., J. Burgfeld, F. Goerke, M. Rennicke, M. Schwanstecher, A. Wallasch, B.J. Zünkler and S. Lenzen, 1989, Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets, Biochem. Pharmacol. 38, 1217.

Plant, T.D. and J.C. Henquin, 1990, Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic B-cells, Br. J. Pharmacol. 101, 115.

Ribalet, B. and P.M. Beigelman, 1981, Effects of divalent cations on β-cell electrical activity, Am. J. Physiol. 241, C59.

Schwanstecher, M., C. Brandt, S. Behrends, U. Schaupp and U. Panten, 1992, Effect of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic β-cell line and rat cerebral cortex, Br. J. Pharmacol. 106, 295.

Sturgess, N.C., M.L.J. Ashford, D.L. Cook and C.N. Hales, 1985, The sulphonylurea receptor may be an ATP-sensitive potassium channel, Lancet 2, 474.

Trube, G., P. Rorsman and T. Ohno-Shozaku, 1986, Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channels in mouse pancreatic beta-cells, Pflüg. Arch. 47, 493.

Yamashita, T., Y. Masuda and S. Tanaka, 1995, The effects of potassium channel openers and blockers on the specific binding sites for [^{3}H]glibenclamide in rat tissues, Jpn. J. Pharmacol. 67, 35.

Zünkler, B.J., S. Lenzen, K. Manner, U. Panten and G. Trube, 1988, Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells, Naunyn-Schmied. Arch. Pharmacol. 337, 225.