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Abstract
Sulfonylureas stimulate insulin secretion by blocking ATP-sensitive K+ channels (K+-ATP channels) of the beta-cell membrane, thereby causing depolarization, Ca2+ influx, and rise in cytoplasmic Ca2+ concentration ([Ca2+]), whereas diazoxide inhibits insulin secretion by opening K+-ATP channels. It has been suggested recently that these drugs also respectively increase and decrease the efficacy of Ca2+ on exocytosis. This hypothesis was tested here with intact islets or single beta-cells from normal mice. Depolarizing islet cells by raising extracellular K+ from 4.8 to 15, 30, and 60 mmol/l progressively raised [Ca2+]i and stimulated insulin secretion. The magnitude of the [Ca2+]i rise produced by a subsequent addition of 100 micromol/l tolbutamide decreased as the concentration of K+ was increased. The effect on insulin secretion paralleled that on [Ca2+]i. Similarly, the magnitudes of the [Ca2+]i drop and of the inhibition of insulin secretion produced by 250 micromol/l diazoxide w...

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Tolbutamide and Diazoxide Influence Insulin Secretion by Changing the Concentration but Not the Action of Cytoplasmic Ca^{2+} in β-Cells

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Sulfonylureas stimulate insulin secretion by blocking ATP-sensitive K⁺ channels (K⁺-ATP channels) of the β-cell membrane, thereby causing depolarization, Ca^{2+} influx, and rise in cytoplasmic Ca^{2+} concentration ([Ca^{2+}]), whereas diazoxide inhibits insulin secretion by opening K⁺-ATP channels. It has been suggested recently that these drugs also respectively increase and decrease the efficacy of Ca^{2+} on exocytosis. This hypothesis was tested here with intact islets or single β-cells from normal mice. Depolarizing islet cells by raising extracellular K⁺ from 4.8 to 15, 30, and 60 mmol/l progressively raised [Ca^{2+}], and stimulated insulin secretion. The magnitude of the [Ca^{2+}] rise produced by a subsequent addition of 100 µmol/l tolbutamide decreased as the concentration of K⁺ was increased. The effect on insulin secretion paralleled that on [Ca^{2+}]. Similarly, the magnitudes of the [Ca^{2+}], drop and of the inhibition of insulin secretion produced by 250 µmol/l diazoxide were inversely related to the concentration of K⁺. Either drug was effective on secretion only when it increased or decreased [Ca^{2+}]. Exocytosis of insulin granules from single, voltage-clamped β-cells was also studied by measuring cell capacitance changes. In the perforated patch configuration, exocytosis was evoked by depolarizing pulses. Addition of tolbutamide to the extracellular medium did not affect the Ca^{2+} current and the resulting change in cell capacitance. In the whole-cell configuration, cell capacitance increased with the concentration of free Ca^{2+} in the solution diffusing from the pipette into the cell. It was markedly potentiated by cAMP, which was inhibited by activation of α₂-adrenoceptors with clonidine, and was strongly augmented by acetylcholine. In contrast, tolbutamide was ineffective whether applied intra- or extracellularly, at low or high free Ca^{2+}, and with or without cAMP. Diazoxide also failed to interfere directly with exocytosis. These results indicate that tolbutamide and diazoxide affect insulin secretion by changing the concentration, not the action, of Ca^{2+} in β-cells.

Glucose stimulates insulin secretion by two mechanisms that are respectively dependent and independent of ATP-sensitive K⁺ channels (K⁺-ATP channels). By closing these channels in the plasma membrane of β-cells, glucose initiates the following sequence of events: membrane depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and rise in cytoplasmic Ca^{2+} ([Ca^{2+}]), which constitutes the triggering signal for exocytosis of insulin granules (1–4). The second mechanism does not involve further changes in [Ca^{2+}], but an increase in the efficacy of Ca^{2+} on exocytosis, through as-yet-unidentified pathways (5–7).

Hypoglycemic sulfonylureas remain the only useful drugs to increase a deficient insulin secretion and, therefore, are a cornerstone of the pharmacological treatment of type 2 diabetes (8). They largely mimic the effects of glucose in the generation of the triggering signal (9–11). However, a major difference between both secretagogues is that the closure of K⁺-ATP channels by sulfonylureas does not result from changes in β-cell metabolism but from a direct interaction with the sulfonylurea receptor (12,13), which is a subunit of the K⁺-ATP channel (14,15). Activation of a Cl⁻ channel and inhibition of the Na⁺ pump might also contribute to the depolarization-mediated rise in [Ca^{2+}], brought about by sulfonylureas (10). Recently, it has been suggested that sulfonylureas also increase insulin release by a second, very different, mechanism which, like that of glucose, does not involve a rise in [Ca^{2+}], but an increase in Ca^{2+} efficacy on exocytosis (16,17).

Diazoxide has long been used to inhibit inappropriate insulin secretion causing hypoglycemia (18). Its major mode of action is the opening of K⁺-ATP channels in the β-cell membrane, with repolarization, closure of voltage-dependent Ca^{2+} channels, and lowering of [Ca^{2+}]i (19–21). However, it has been proposed that diazoxide also decreases the efficacy of Ca^{2+} on exocytosis (16,22).

These proposals that sulfonylureas and diazoxide can influence insulin secretion without necessarily changing [Ca^{2+}]i in β-cells are based on two types of experiments. First, tolbutamide and glibenclamide were found to increase, whereas diazoxide was found to decrease, insulin release from permeabilized RINm5F or islet cells in which the membrane potential was dissipated and the composition of the intracellular milieu, including [Ca^{2+}]i, was clamped (16). Second, tolbutamide was found to increase and diazoxide to decrease exocytosis of insulin granules estimated by changes in membrane capacitance of voltage-clamped β-cells (17,22).

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ACh, acetylcholine; [Ca^{2+}], cytoplasmic Ca^{2+} concentration; C_{0m}, cell membrane capacitance; G_{m}, access conductance; G_{m'}, cell membrane conductance; K⁺-ATP channel, ATP-sensitive K⁺ channel.

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In contrast with these results, we previously failed to confirm that tolbutamide and glibenclamide are effective secretagogues in permeabilized islet cells and to find evidence that they can increase insulin secretion from intact islets when they do not increase \([\text{Ca}^{2+}]_i\) (23).

In this study, we have pursued the investigation of the question for several reasons. First, it is not known whether the newly proposed mechanism of inhibition of insulin secretion by diazoxide also exists in intact cells. Second, it is crucial to know whether sulfonylureas have one or two modes of action and, therefore, whether they may be expected to partially or completely correct defects in glucose regulation of insulin release. Third, identification of a K+ATP channel-independent mode of action of sulfonylureas and diazoxide would considerably help in elucidating the second pathway of regulation by glucose. The experiments were performed with normal mouse islets in which the effects of tolbutamide and diazoxide on \([\text{Ca}^{2+}]_i\) and insulin secretion were compared. The effects of the drugs on exocytosis were also estimated by recording capacitance changes in single β-cells, the electrophysiological technique that was previously used to identify their novel mode of action (17,22).

RESEARCH DESIGN AND METHODS

Preparation. Islets were isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), followed by hand-picking. They were then cultured in RPMI 1640 medium (Gibco BRL, Paisley, Scotland) supplemented with 10 mmol/l glucose, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. For patch-clamp experiments, the islets were dispensed into single cells with a Ca2+-free buffer containing 100 µmol/l EGTA and 100 µg/ml trypsin. The cells were then cultured in the same medium as above. β-cells make up about 80% of islet cells in normal mice (24) and have a larger diameter (11–14 µm) than δ-cells (8–11 µm) (25). The cells used for the electrophysiological experiments were selected for their large size. From their basal capacitance (~ 6 pF) and a specific membrane capacitance of 10 fF/µm2, one can recalculate that these cells had a diameter of about 13–14 µm.

Solutions. The medium used for islet isolation was a bicarbonate-buffered solution that contained 120 mmol/l NaCl, 4.5 mmol/l KCl, 2.5 mmol/l CaCl2, 1.2 mmol/l MgCl2, and 24 mmol/l NaHCO3. It was gassed with O2/CO2 (94:6) to maintain pH 7.4 and was supplemented with bovine serum albumin (1 mg/ml). A similar medium was used for measurements of \([\text{Ca}^{2+}]_i\) and insulin secretion. When the concentration of KCl was increased to 15, 30, or 60 mmol/l, that of NaCl was decreased accordingly to maintain iso-osmolality. When the concentration of CaCl2 was decreased to 0.5 mmol/l, that of MgCl2 was increased from 1.2 to 3.2 mmol/l. Tolbutamide (Sigma, St. Louis, MO) and diazoxide (Schering-Plough, Kiels, Germany) were dissolved in the medium. Batches of 20 islets were then placed in parallel perfusion chambers and perifused at a flow rate of 1.2 ml/min (36). Effluent fractions collected at 2-min intervals were chilled until their insulin content was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark).

Measurements of β-cell membrane capacitance. Patch-clamp experiments were performed at 35°C in the conventional whole-cell recording configuration or in perforated mode, using an EPC9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). Cultured cells were first preincubated for 30 min in the presence of 10 µmol/l forskolin and then perfused at a rate of 1 ml/min. The bath and pipette solutions were as described above. Patch pipettes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, U.K.) to give a resistance of 2–3 MΩ after coating with Silgard (Dow Corning, Wiesbaden, Germany) and firepolished. For recordings in perforated-patch configuration, pipettes were dipped for a few seconds in the pipette solution free of amphotericin B and then back-filled with the same solution containing 250 µg amphotericin B/ml. Perforation usually occurred progressively, in less than 5 min. The access conductance was considered large enough to begin capacitance measurements when exceeding 30 nS.

To assay exocytosis, membrane capacitance was measured with a technique derived from the phase-sensitive method (27). A 509-Hz sine wave with a peak-to-peak amplitude of 50 mV was applied to the cell under voltage-clamp conditions (~70 mV), and the resulting membrane current was filtered at 3 kHz with a 4-pole Bessel filter. The membrane capacitance \((C_m)\) and conductance \((G_m)\) and the access conductance \((G_a)\) were derived from analysis of the sinusoidal membrane current at two orthogonal phase angles. This was done with the Lockin extension of the Pulse software (HEKA Electronics) using the “sine + dc” protocol derived from the method referred to as “computer-aided reconstruction of passive cell parameters” by Lindau and Neher (28,29). The reversal potential of the DC current was assumed to be 0. Protocols of 8 cycles (9.6 ms in total) were applied to the cell every 100 ms. A single value of each membrane parameter \((C_m, G_m, G_a)\) was computed each cycle; an average value was calculated from the 8 values and eventually sent to the Xchart Software (HEKA Electronics). From an initial sampling frequency of 50 kHz for the sinusoidal membrane current, this gave a final frequency acquisition of 10 Hz for capacitance measurements using Xchart.

In the perforated mode, exocytosis was triggered by applying voltage steps. Every 2 min, the Xchart trace was temporarily interrupted to apply a voltage pulse from Pulse 8.09 software (HEKA Electronics) using the “sine + dc” protocol derived from the method referred to as “computer-aided reconstruction of passive cell parameters” by Lindau and Neher (28,29). The reversal potential of the DC current was assumed to be 0. Protocols of 8 cycles (9.6 ms in total) were applied to the cell every 100 ms. A single value of each membrane parameter \((C_m, G_m, G_a)\) was computed each cycle; an average value was calculated from the 8 values and eventually sent to the Xchart Software (HEKA Electronics). From an initial sampling frequency of 50 kHz for the sinusoidal membrane current, this gave a final frequency acquisition of 10 Hz for capacitance measurements using Xchart.

In the perforated mode, exocytosis was triggered by applying voltage steps. Every 2 min, the Xchart trace was temporarily interrupted to apply a voltage pulse from Pulse 8.09. This protocol was composed of a control period of 50 ms during which \(C_m, G_m, G_a\) were measured every cycle, a voltage step to 0 mV for 500 ms, and a 1-s period during which \(C_m, G_m, G_a\) were measured every cycle again. This gave a high temporal resolution of \(C_m, G_m, G_a\) for the membrane current during the whole protocol. Membrane capacitance increases after depolarization were calculated 1 s after the end of the voltage pulse to avoid contamination by gating charge movements or tail currents. Then measurements with Xchart at a low temporal resolution (10 Hz) were automatically resumed. Only traces analyzed from Xchart at a low temporal resolution are shown in this study.

In standard whole-cell recording, exocytosis was stimulated by the high concentration of free Ca2+ (0.2–1.25 µmol/l) in the medium diffusing from the pipette into the cell interior. Measurements of \(C_m\) were performed with the above protocol as long as the cell secreted and until \(C_m\) reached a plateau.

The exocytotic responses were estimated as maximum changes in cell capacitance \((\Delta C_m)\) and, for whole-cell recordings, as rates of capacitance changes \((\Delta C_m/\Delta t)\) calculated during the first min or between 20 and 80% of the \(C_m\) variation induced by the stimulus.

Statistical analysis. Results are usually presented as means ± SE. For [Ca2+]i measurements, recordings were obtained with five or six individual islets from each preparation. The same experiment was repeated with three or four distinct islet preparations. For insulin release, each protocol was tested with islets from six to nine different preparations. For electrophysiological recordings, experiments were repeated on two to four different batches of cells. Results were compared with a paired or unpaired t test, according to the type of experiment.

RESULTS

Effects of tolbutamide on islet \([\text{Ca}^{2+}]_i\) and insulin secretion. The experiments were carried out in the presence of variable concentrations of KCl to produce different degrees of β-cell depolarization (30) before stimulation with
tolbutamide. In a control medium containing 4.8 mmol/l K⁺, 15 mmol/l glucose induced oscillations of [Ca²⁺] in the islets (21), which cannot be seen in Fig. 1 (upper left panel) because recordings from different islets have been averaged. Addition of 100 µmol/l tolbutamide caused a rapid and sustained rise in [Ca²⁺] that was completely reversible upon removal of the sulfonylurea. Under these conditions, tolbutamide reversibly doubled glucose-induced insulin secretion (Fig. 1, upper right panel).

When the medium contained 15 instead of 4.8 mmol/l K⁺, [Ca²⁺] and insulin secretion were elevated, but tolbutamide remained effective on both [Ca²⁺] and secretion (Fig. 1, mid-
dle panels). In contrast, tolbutamide only produced a small and transient further rise in \([Ca^{2+}]_{i}\) and no change in insulin secretion in the presence of 30 mmol/l K\(^+\), and it was completely ineffective in the presence of 60 mmol/l K\(^+\) (Fig. 1, lower panels).

To determine whether this lack of effect is due to membrane depolarization or to high \([Ca^{2+}]_{i}\), the effects of tolbutamide were also tested in a medium containing 60 mmol/l K\(^+\) and 0.5 instead of 2.5 mmol/l CaCl\(_2\) (Fig. 1, lower panels). Under these conditions, both \([Ca^{2+}]_{i}\) and the insulin secretion rate were between those recorded in the presence of 4.8 and 15 mmol/l K\(^+\), but tolbutamide was again without effect. Similar experiments (n = 7) were also performed in the presence of 0.5 mmol/l dibutyryl cAMP. Control \([Ca^{2+}]_{i}\) was not affected, but control insulin secretion was increased by ~80% (293 ± 39 vs. 162 ± 16 pg · islet\(^-1\) · min\(^-1\)). This, however, did not disclose any effect of tolbutamide (not shown).

**Effects of tolbutamide on \(\beta\)-cell capacitance changes studied in intact \(\beta\)-cells.** Whole-cell \(Ca^{2+}\) current and cell capacitance were measured using the perforated-patch, whole-cell configuration. The mean basal cell capacitance averaged 6.89 ± 0.31 pF. Under control conditions, 500-ms depolarizations from –70 to 0 mV evoked \(Ca^{2+}\) currents of about 200 pA at the peak and capacitance increases that averaged about 80 fF (Fig. 2A). When the stimulation was repeated every 2 min up to 10 min after the addition of 100 µmol/l tolbutamide to the bath, the amplitude of the \(Ca^{2+}\) current remained fairly stable, and the evoked capacitance change was not significantly affected (Fig. 2B). In contrast, the increase in capacitance was tripled after 6 min of treatment with 25 nmol/l phorbol 12-myristate 13-acetate to activate protein kinase C (not shown).

**Validation of the whole-cell configuration to study exocytosis by cell capacitance.** The mean initial cell capacitance \((C_m)\) recorded upon rupture of the \(\beta\)-cell membrane averaged 6.89 ± 0.31 pF. Under control conditions, 500-ms depolarizations from –70 to 0 mV evoked \(Ca^{2+}\) currents of about 200 pA at the peak and capacitance increases that averaged about 80 fF (Fig. 2A). When the stimulation was repeated every 2 min up to 10 min after the addition of 100 µmol/l tolbutamide to the bath, the amplitude of the \(Ca^{2+}\) current remained fairly stable, and the evoked capacitance change was not significantly affected (Fig. 2B). In contrast, the increase in capacitance was tripled after 6 min of treatment with 25 µmol/l phorbol 12-myristate 13-acetate to activate protein kinase C (not shown).
both its extent and rate were augmented by the presence of 100 µmol/l acetylcholine (ACh) in the extracellular solution (Fig. 3B). In contrast, the response evoked by 1.25 µmol/l free Ca\(^{2+}\) was strongly inhibited by clonidine (Fig. 3C). These results thus show that exocytosis studied with this technique is Ca\(^{2+}\) dependent, increased by cAMP, and sensitive to potentiation and inhibition by activation of muscarinic and \(\alpha_2\)-adrenergic receptors, respectively.

**Effects of tolbutamide on cell capacitance in the presence of 0.2 µmol/l intracellular Ca\(^{2+}\).** When \(\beta\)-cells were dialysed with a pipette solution containing 0.2 µmol/l Ca\(^{2+}\) and 1 mmol/l cAMP, an increase in cell capacitance averaging about 1 pF was recorded (Fig. 4A). The presence of 100 µmol/l tolbutamide in the pipette solution or in the bath solution (which was devoid of CaCl\(_2\)) had no effect on the maximum change in cell capacitance. The rate of change in cell capacitance (d\(C_m\)/dt) was also unaffected by tolbutamide, whether it was calculated between 20 and 80% of the changes (Fig. 4A, bottom) or during the 1st min of change: 6.6 ± 1.2, 7.5 ± 1.6, and 6.0 ± 1.6 fF/s for controls, To lb\(_b\), or To lb\(_p\), respectively. Unlike ACh, tolbutamide was also ineffective when added to the bath after the increase in cell capacitance had developed in response to Ca\(^{2+}\) and cAMP (Fig. 4B, bottom). When the experiments were carried out in the presence of 0.2 µmol/l Ca\(^{2+}\) and the absence of cAMP, cell capacitance did not significantly change in the absence or presence of tolbutamide in the pipette (Fig. 4B).

**Effects of tolbutamide on cell capacitance in the presence of 1.25 µmol/l intracellular Ca\(^{2+}\).** In control cells, the maximum increase and the rate of change in cell capacitance evoked by 1.25 µmol/l Ca\(^{2+}\) augmented with the concentration of cAMP, but they were not significantly affected by the presence of 100 µmol/l tolbutamide in the pipette solution (Figs. 5A and 5B). Addition of tolbutamide to the bath solution was also ineffective under these conditions (data not shown).

**Effects of diazoxide on \([Ca^{2+}]_i\) in islet cells and insulin secretion.** In the presence of 4.8 mmol/l K\(^+\) and 15 mmol/l glucose, 250 µmol/l diazoxide lowered [Ca\(^{2+}\)], and the insulin secretion rate to basal levels (Fig. 6, upper panels). Decreases in [Ca\(^{2+}\)] and insulin secretion were also observed when diazoxide was added to a medium containing 15 or 30 mmol/l K\(^+\). Diazoxide is known to repolarize the \(\beta\)-cell mem-

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**FIG. 3.** Influence of cAMP, acetylcholine, and clonidine on the changes in cell capacitance evoked by dialysis of \(\beta\)-cells with 0.2 or 1.25 µmol/l free Ca\(^{2+}\). The upper panels illustrate representative increases in cell capacitance occurring after establishment of the whole-cell recording configuration. cAMP was present in the pipette solution, whereas ACh and clonidine were present in the bath solution (for ~5 min before breaking of the membrane). The middle panels show the average changes in capacitance (\(\Delta C_m\)), and the lower panels show the average rates of capacitance changes (d\(C_m\)/dt) calculated between 20 and 80% of the total change. Values are means ± SE for 10–17 cells. The access conductance (\(G_a\)) decreased with time from an average initial value of 119 ± 5 nS to 86 ± 5 nS. A change of \(G_a\) within this range of values does not significantly affect calculation of \(C_m\) (29).
brane partially under these conditions (5). In contrast, neither [Ca\(^{2+}\)] nor insulin secretion was affected by diazoxide in the presence of 60 mmol/l K\(^+\), irrespective of the Ca\(^{2+}\) concentration in the medium and, hence, of the degree of β-cell stimulation (Fig. 6, lower panels).

**Effects of diazoxide on cell capacitance in the presence of 1.25 μmol/l intracellular Ca\(^{2+}\).** The presence of 250 μmol/l diazoxide in the pipette solution was without effect on the maximum change (ΔC\(_{\text{m}}\)) in cell capacitance evoked by 1.25 μmol/l Ca\(^{2+}\) in the presence of 1 mmol/l cAMP (Fig. 5C). The rate of change in cell capacitance (dC\(_{\text{m}}\)/dt) was also unaffected by diazoxide, whether it was calculated between 20 and 80% of the changes (Fig. 5C, bottom) or during the 1st min of change: 12.9 ± 2.7 and 11.9 ± 1.7 fF/s without and with diazoxide, respectively.

**DISCUSSION**

There is no dispute that K\(^+\)-ATP channels are the primary site of action of tolbutamide and diazoxide in β-cells. Their closure and opening eventually increase and decrease [Ca\(^{2+}\)]\(_{\text{i}}\), leading to stimulation and inhibition of insulin secretion, respectively. The question addressed in this study was whether these drugs also affect secretion by modulating the action of Ca\(^{2+}\) on the exocytic process.

To test the hypothesis, it is essential that [Ca\(^{2+}\)]\(_{\text{i}}\) remains stable because any change might account for an accompanying change in secretion. This is not easy in intact islets, the use of which is, however, essential to ascertain the physiological relevance of observations made with indirect approaches. When islets are studied in a control medium (4.8 mmol/l K\(^+\), 15 mmol/l glucose), the blockade of K\(^+\)-ATP channels by tolbutamide causes depolarization, rise in [Ca\(^{2+}\)]\(_{\text{i}}\), and stimulation of insulin release (9–12). The latter two events were measured in this study. Raising the concentration of K\(^+\) in the medium to depolarize the β-cell membrane (30) increased control [Ca\(^{2+}\)]\(_{\text{i}}\), and insulin release, but it caused a parallel attenuation of the effects of tolbutamide on both [Ca\(^{2+}\)]\(_{\text{i}}\) and secretion. That the eventual abrogation of tolbutamide action on secretion results from an inability to raise [Ca\(^{2+}\)]\(_{\text{i}}\), was demonstrated by the experiments performed in 60 mmol/l K\(^+\) and 0.5 mmol/l CaCl\(_2\) (10). Tolbutamide was inactive in these strongly depolarized islets, although [Ca\(^{2+}\)]\(_{\text{i}}\), was similar to that of islets that were only partially depolarized by 15 mmol/l K\(^+\) (in the presence of 2.5 mmol/l CaCl\(_2\)) and in which the drug still raised [Ca\(^{2+}\)]\(_{\text{i}}\), and secretion.

Exocytosis can be studied at the single-cell level by monitoring changes in cell capacitance (27–29). Because cell capacitance is proportional to cell surface area, and because the latter increases after fusion with secretory vesicles during exocytosis, secretion is detected by increases in cell capacitance. Conversely, endocytosis is followed by decreases in cell capacitance, and the measured changes are the net result of both processes. The technique has already been successfully applied to studies of insulin secretion from single β-cells (34–38).

With the perforated-patch technique, the cell remains intact and exocytosis is triggered by depolarizing pulses that activate Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels. These experiments were carried out in the presence of forskolin because the capacitance changes evoked by Ca\(^{2+}\) influx are small if cAMP levels are low in β-cells (35,36; P.M., unpublished observations). Under these conditions, depolarizations for 500 ms to 0 mV evoked increases in cell capacitance similar to those reported by others (17,35,36). However, tolbutamide failed to increase the response, which contrasts with the doubling of the response that a similar concentration of the sulfonylurea produced in another study using similar tissue and conditions (17).

Cell capacitance changes can also be measured in the standard whole-cell configuration (37,38). After rupture of the plasma membrane at the tip of the recording pipette, the solution contained in the pipette diffuses within the cell and, if appropriate, stimulates exocytosis. Our results show that cell capacitance increased with the concentration of free Ca\(^{2+}\) (38), was markedly potentiated by cAMP, was inhibited by activation of α₂-adrenoceptors with clonidine (39), and was strongly augmented by acetylcholine. In spite of this evidence that our experimental model functions adequately, being able to detect receptor-mediated changes in exocytosis, tolbutamide was again found to be without effect. In a recent study, as yet reported in abstract form only (22), intracellular application of 100 μmol/l tolbutamide did not influence the strong stimulation of exocytosis evoked by 2 μmol/l Ca\(^{2+}\) in the presence of cAMP. Our experiments with 1.25 μmol/l Ca\(^{2+}\), two concentrations of cAMP, and intracellular tolbutamide confirm this lack of effect. However, neither the maximum change in capacitance nor the rate...
of change was significantly affected by tolbutamide when we tested the drug in the presence of 200 nmol/l Ca\(^{2+}\), without or with cAMP. This contrasts with the report (22) that tolbutamide augmented the initial (~60 s) rate of exocytosis in the presence of 170 nmol/l Ca\(^{2+}\), with or without cAMP. Whether the drug had a net effect on exocytosis (total change in capacitance) was not reported in the abstract (22).

Like those of tolbutamide, the effects of diazoxide on insulin secretion from intact islets have been studied during perifusions with solutions containing various K\(^{+}\) concentrations. The drug was found to inhibit secretion as long as it also decreased [Ca\(^{2+}\)]\(_{\text{i}}\) in islet cells. If diazoxide were able to interfere with the action of Ca\(^{2+}\) on exocytosis, it should have inhibited insulin secretion in the presence of 60 mmol/l K\(^{+}\) even when it failed to lower the moderately (0.5 mmol/l extracellular CaCl\(_2\)) or markedly (2.5 mmol/l extracellular CaCl\(_2\)) elevated [Ca\(^{2+}\)]\(_{\text{i}}\). Our measurements of cell capacitance also failed to disclose any direct inhibition of the exocytotic process by diazoxide applied intracellularly. Thus, they disagree with a previous study reporting that diazoxide strongly reduces the rate of capacitance change induced by tolbutamide or Ca\(^{2+}\) (22). It should also be borne in mind that many laboratories have reported that, provided glucose is present, high K\(^{+}\) stimulates a sustained release of insulin from intact islets in the presence of diazoxide (7,40–42). This would not be so if diazoxide had any strong inhibitory action on exocytosis.

What can be the reason for the disparity between the present and previous results (17,22) based on capacitance measurements? With the reservation that few technical details are given in an abstract (22), comparison of both approaches shows the following important similarities and potentially significant differences. The animal species, the recording temperature, and the concentrations of tolbutamide and forskolin were identical, and the concentration of diazoxide was only slightly different (250 vs. 400 µmol/l). Our solutions contained 20 or 5 mmol/l glucose as compared with 5 or 0 mmol/l glucose (17), but this was not found to make any difference in either study. Finally, two different algorithms were used for capacitance measurements. The technique of Eliasson et al. (17) is based on the classic phase-sensitive method (43), whereas ours is based on a modification of this method (28) that has recently proved to be very reliable with the “Pulse-lockin” EPC-9 software that we used (44). Discussion of the respective advantages and limitations of each technique is beyond the scope of this paper, in particular because both approaches have usually been found to yield qualitatively similar results in other systems. We thus have no explanation for the disparity between our results and those of others, but we insist on the fact that our conclusions rely on data obtained with several methodologies.

By using three approaches to study insulin secretion, direct measurements from intact islets perifused for several minutes or from incubated permeabilized islet cells (23) and indirect estimations of exocytosis by recording cell capacitance changes during a few seconds or minutes, we conclude that tolbutamide and diazoxide affect secretion by changing the concentration, not the action, of Ca\(^{2+}\) in \(\beta\)-cells. If the latter effect exists, as suggested by others (16,17,22), it must be short-lived and small enough to escape our detection, and it is unlikely to be therapeutically relevant. There is no evidence that sulfonylureas mimic the second mechanism by which glucose stimulates insulin secretion (5–7). This justifies ongoing searches for new drugs acting on \(\beta\)-cells.

FIG. 5. Lack of effect of tolbutamide and diazoxide on the changes in cell capacitance evoked by dialysis of \(\beta\)-cells with 1.25 µmol/l Ca\(^{2+}\) and 0.1 or 1 mmol/l cAMP. The upper traces illustrate representative changes in cell capacitance occurring after establishment of the whole-cell recording configuration. cAMP, tolbutamide (100 µmol/l), and diazoxide (250 µmol/l) were present in the pipette solution (Tolb\(_1\), Dz\(_1\)). The other panels show average changes in capacitance and rate of capacitance changes, as in Fig. 3. Values are means ± SE for 9–14 cells.
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