PKC-Dependent Stimulation of Exocytosis by Sulfonylureas in Pancreatic β Cells

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Hypoglycemic sulfonylureas represent a group of clinically useful antidiabetic compounds that stimulate insulin secretion from pancreatic β cells. The molecular mechanisms involved are not fully understood but are believed to involve inhibition of potassium channels sensitive to adenosine triphosphate (K_{ATP} channels) in the β cell membrane, causing membrane depolarization, calcium influx, and activation of the secretory machinery. In addition to these effects, sulfonylureas also promoted exocytosis by direct interaction with the secretory machinery not involving closure of the plasma membrane K_{ATP} channels. This effect was dependent on protein kinase C (PKC) and was observed at therapeutic concentrations of sulfonylureas, which suggests that it contributes to their hypoglycemic action in diabetics.

Sulfonylureas, such as tolbutamide, stimulate insulin secretion by inhibiting ATP-sensitive K^+ channels (K_{ATP} channels) in the β cell plasma membrane (*1*). A 140-kD high-affinity sulfonylurea receptor has been purified and cloned (*2*), which associates with a β cell inward rectifier to form functional K_{ATP} channels (*3*). However, it seems possible that sulfonylurea receptors have additional cellular functions. For example, 80 to 90% of the sulfonylurea binding proteins are localized to intracellular membranes, including those of the secretory granules (*4*). Here we demonstrate, using capacitance measurements of exocytosis (*5*), that sulfonylureas can potentiate exocytosis of insulin-containing granules by a mechanism exerted distally to the closure of the K_{ATP} channels.

We measured whole-cell Ca^{2+} current and cell capacitance (*6*) in intact pancreatic β cells, using the perforated patch whole-cell configuration. Under control conditions, a 500-ms depolarization to 0 mV evoked a step capacitance increase of 18 fF (Fig. 1A). Six minutes after the addition of 100 μM tolbutamide, the same depolarization evoked a threefold larger capacitance increase of 52 fF while not affecting the amplitude of the Ca^{2+} current. On average, tolbutamide increased exocytosis 2.5-fold (+150 ± 50% above control values; n = 11, $P < 0.02$) (Fig. 1B). This stimulation of exocytosis could not be accounted for by any depolarizing action of tolbutamide because the cell was voltage-clamped and the membrane potential held at −70 mV regardless of K_{ATP} channel activity.

Tolbutamide remained equally stimulatory when exocytosis was already potentiated by forskolin (Fig. 1, C and D), which stimulates exocytosis by a mechanism dependent on cyclic adenosine monophosphate and protein kinase A (PKA) (*7*). On average, tolbutamide doubled the exocytotic response (+105 ± 40% above control values; n = 6, $P < 0.05$) in the presence of forskolin.

The ability of tolbutamide to enhance secretion was shared by the more novel (second generation) sulfonylurea glibenclamide (Fig. 1E). On average, glibenclamide (0.1 μM) produced a threefold potentiation of the exocytotic responses (increase of 205 ± 75%; $P < 0.05$) over 6 min without affecting the amplitude of the Ca^{2+} current. Under conditions precluding further membrane depolarization, glibenclamide stimulated insulin secretion to an equal extent (*8*). Glipizide (1 μM) produced a comparable (≈2.5-fold) potentia-

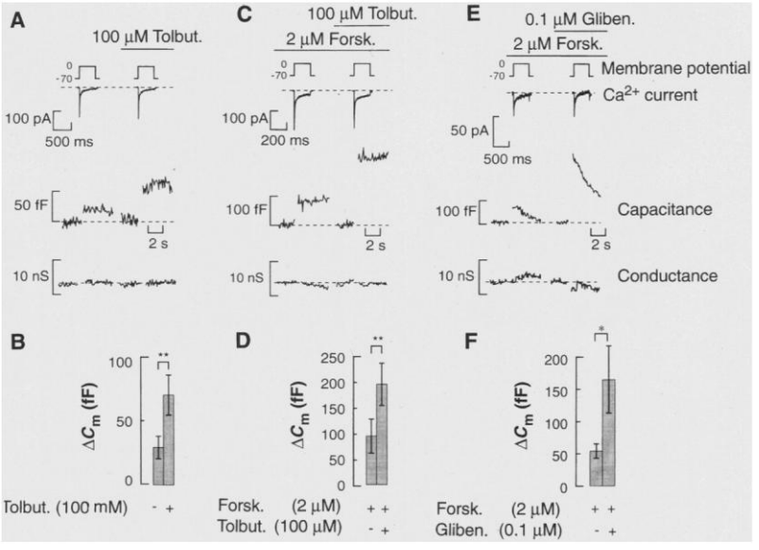

Fig. 1. (**A**) Ca^{2+} currents and changes in cell capacitance and membrane conductance (*18*) evoked by a 500-ms depolarization before (left) and 6 min after (right) the addition of 100 μM tolbutamide recorded from the same cell. (**B**) Average effect of tolbutamide on the capacitance increase (ΔC_m) elicited by the voltage-clamp depolarization in 11 cells. (**C**) Ca^{2+} currents and changes in cell capacitance and membrane conductance evoked by a 200-ms depolarization before and after addition of 100 μM tolbutamide in the presence of 2 μM forskolin. (**D**) Average effect of tolbutamide in the presence of forskolin on the capacitance increase in four different cells. (**E**) Ca^{2+} currents and changes in cell capacitance evoked by 500-ms depolarizations applied before and 6 min after the addition of 0.1 μM glibenclamide. The gradual decrease in cell capacitance seen after the depolarization-induced increase likely reflects endocytosis of secreted membranes, which in this cell has unusually rapid kinetics. (**F**) Average effect of glibenclamide in five different cells. The single asterisk indicates $P < 0.05$; the double asterisks indicate $P < 0.02$. Forsk., forskolin; Gliben., glibenclamide; Tolbut., tolbutamide.

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tion of exocytosis, from a basal 60 ± 34 fF to 145 ± 72 fF 6 min after its addition ($P < 0.05$). Diazoxide (0.4 mM), a sulfonamide chemically related to the sulfonylureas, was ineffective.

The stimulatory action of tolbutamide was reversibly abolished by the PKC inhibitor bisindolylmaleimide (Fig. 2, A and B) (9). Tolbutamide also lacked stimulatory action when PKC was fully activated by exposure of the β cell to 10 nM PMA (phorbol 12-myristate 13-acetate) (*10*). These observations suggest that the stimulatory action of tolbutamide depends on the activity of PKC. By contrast, activation of PKA is clearly not required for the stimulatory effect of the sulfonylureas; they still more than doubled the exocytotic response (+110 ± 30% above the control; $P < 0.025$, $n = 6$) in the presence of 10 μM Rp-cAMPS (adenosine-3′,5′-cyclic monophosphothioate, Rp-isomer), an inhibitor of PKA [Fig. 2, C and D; compare (*7*)].

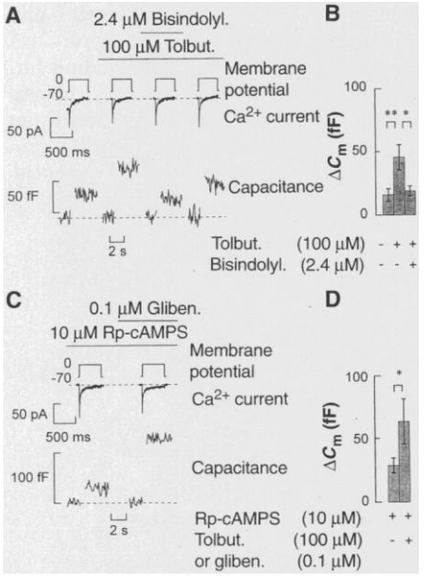

Fig. 2. (**A**) Ca^{2+} currents and changes in cell capacitance evoked by a 500-ms depolarization under control conditions, 4 min after the addition of 100 μM tolbutamide, 2 min after addition of 2.4 μM bisindolylmaleimide (Calbiochem, LaJolla, California) in the continued presence of tolbutamide, and 6 min after removal of bisindolylmaleimide. (**B**) Average effects of tolbutamide and bisindolylmaleimide on the capacitance increase (ΔC_m) elicited by the voltage-clamp depolarization in six cells. (**C**) Ca^{2+} currents and changes in cell capacitance evoked by a 500-ms depolarization before and 6 min after the addition of 0.1 μM glibenclamide in B cells pretreated (>15 min) with 10 μM Rp-cAMPS (a PKA inhibitor) (*19*) (Biolog, Hamburg). (**D**) Average effect of sulfonylureas (glibenclamide or tolbutamide) on capacitance increases evoked by 500-ms depolarizations in the presence of Rp-cAMPS in six different cells. The single asterisk indicates $P < 0.05$; the double asterisks indicate $P < 0.02$.

The stimulatory action of the sulfonylureas on exocytosis was not the result of the voltage-gated Ca^{2+} currents being increased with resultant stimulation of Ca^{2+}-dependent exocytosis (Fig. 3A). Simultaneous measurements of voltage-clamp Ca^{2+} currents, cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) (*11*), and cell capacitance revealed that 100 μM tolbutamide stimulated exocytosis without affecting depolarization-evoked $[Ca^{2+}]_i$ transients (Fig. 3B). Moreover, neither the peak nor the integrated Ca^{2+} current was affected by tolbutamide or other sulfonylureas (Fig. 3C).

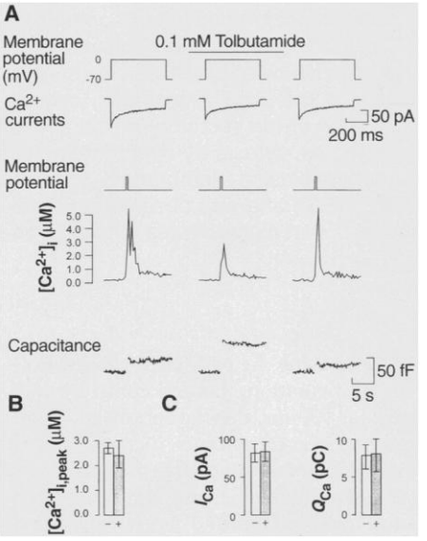

Fig. 3. (**A**) Ca^{2+} currents and changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by 500-ms depolarization before and 8 min after addition of 0.1 mM tolbutamide and 6 min after withdrawal of tolbutamide. The experiment was conducted in the presence of 2 μM forskolin. (**B**) Mean amplitude of depolarization-evoked $[Ca^{2+}]_i$ transients before (−) and 6 min after (+) the addition of 0.1 mM tolbutamide in four separate experiments. (**C**) Lack of effect of sulfonylureas on peak (I_{Ca}) and integrated (Q_{Ca}) Ca^{2+} currents determined before (−) and 6 min after (+) the addition of the sulfonylureas (glibenclamide, glipizide, or tolbutamide) in 12 separate experiments (four experiments with each of the sulfonylureas).

The effect of tolbutamide on exocytosis was dependent on dose (Fig. 4). No stimulation of exocytosis was observed at concentrations ≤10 μM. At higher concentrations, tolbutamide reversibly potentiated exocytosis by 40 to 100% (Fig. 4, inset). Approximating the percentage stimulation of exocytosis observed at the different concentrations of tolbutamide to the Hill equation (Fig. 4) yielded values of the association constant (K) and cooperativity factor (n) of 32 μM and 2.5, respectively. These values are somewhat higher than the corresponding values for tolbutamide-induced inhibition of K_{ATP} channel activity in pancreatic β cells (<10 μM and 1, respectively) (*12*).

The mechanisms by which sulfonylureas stimulate exocytosis remain unclear. The sulfonylureas may control exocytosis in β cells by interfering with ionic conductances in the granular membrane, as proposed for pancreatic zymogen granules (*13*), which would be consistent with the fact that 90% of the sulfonylurea receptors localize to the granular membranes (*4*). Members of the ATP-binding cassette superfamily, to which the cloned sulfonylurea receptor (SUR) belongs, may be regulators of channels and pumps (*14*). Thus, we speculate that SUR constitutes a functional part of either a regulatory exocytotic protein or the ion channel complexes in the secretory granule membranes in a way analogous to that demonstrated for the K_{ATP} channel (*3*). The PKC dependence of sulfonylurea action on exocytosis may be explained by the presence of 20 potential PKC phosphorylation sites in SUR (*3*).

In the absence of any information concerning the electrophysiological properties

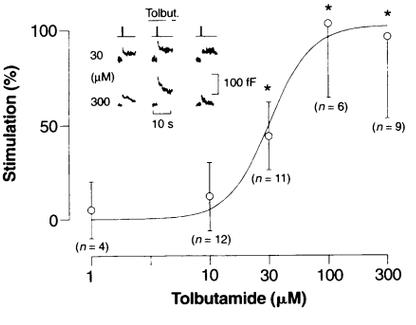

Fig. 4. Concentration dependence of stimulatory action of tolbutamide on exocytosis. Single β cells exposed to forskolin were exposed to 1 μM, 10 μM, 30 μM, 100 μM, or 300 μM tolbutamide. The mean amplitude of the exocytotic responses evoked by two successive depolarizations (500 ms to 0 mV) applied at 2-min intervals in the absence of tolbutamide was taken as the control value. Stimulation of exocytosis by tolbutamide is expressed as the relative increase above the control level as there was significant cell-to-cell variability. The effects were determined 6 min after the addition of tolbutamide to allow a steady state to be attained. Only one concentration was tested in each cell. The curve represents a least-squares fit of the data points to the equation $E/E_{max} = C^n/(C^n + K^n)$, where E is the stimulation of exocytosis observed at the concentration C of tolbutamide, E_{max} is the (calculated) maximum stimulation, K is the dissociation constant, and n is the cooperativity factor. The number of experiments is indicated within the parentheses; the asterisk indicates $P < 0.05$. The inset shows the capacitance responses elicited by the depolarizations before and 6 min after the addition of 30 μM and 300 μM tolbutamide and 4 min after withdrawal of tolbutamide. The vertical lines above the capacitance traces indicate depolarizations (500 ms to 0 mV) applied to trigger exocytosis.

of human diabetic pancreatic β cells, it is not possible to assess directly the relative importance of the different cellular actions of the sulfonylureas on the hypoglycemic action of these compounds in vivo. Our data suggest that in a depolarized β cell, the direct effect of the sulfonylureas on exocytosis may account for as much as 75% of the total stimulatory action (8). This figure would represent the maximum contribution because in β cells that are not voltage-clamped, the sulfonylureas will also stimulate secretion by producing membrane depolarization, acceleration of Ca^{2+} influx, and enhancement of Ca^{2+}-dependent exocytosis (15). The effects of tolbutamide on exocytosis can be observed at concentrations only slightly higher than those required to block the K_{ATP} channels and that are within the therapeutic range (16). The potentiation of exocytosis we report here is therefore likely to contribute to the insulinotropic and hypoglycemic actions of the sulfonylureas in diabetic patients.

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5. Whole-cell currents were elicited by depolarizations to 0 mV from individual tissue-cultured mouse β cells with the use of the perforated patch whole-cell configuration [M. Lindau and J. M. Fernandez, *Nature* **319**, 150 (1986); R. Horn and A. Marty, *J. Gen. Physiol.* **92**, 145 (1988)]. Exocytosis was monitored as changes in membrane capacitance [E. Neher and A. Marty, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6712 (1982); C. Joshi and J. Fernandez, *Biophys. J.* **53**, 885 (1988)]. Depolarizations were applied at 2-min intervals to avoid depression of the exocytotic responses. The extracellular medium consisted of 118 mM NaCl, 20 mM tetraethylammonium chloride, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 5 mM Hepes (pH 7.4 with NaOH). Glucose was usually present at a concentration of 5 mM, but similar effects were observable in its absence. Forskolin, PMA, and most pharmacological agents were dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.01 to 0.1%) and were included in the extracellular medium as indicated. It was ascertained that these concentrations of DMSO do not interfere with the exocytotic responses. The pipettes were filled with 76 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.35 with CsOH). Electrical contact was established by insertion of the pore-forming antibiotic amphotericin B [J. Rae *et al.*, *J. Neurosci. Methods* **37**, 15 (1991)] (final concentration, 0.24 mg/ml) to the pipette solution. All electrophysiological experiments were done at 32° to 34°C. Data are presented as mean values ± SEM of the stated number of experiments, and statistical evidence was evaluated with the Student's *t* test for paired data with each cell serving as its own control.
6. An increase in capacitance can be equated to exocytosis (17) and is unlikely to result from charge movements that occur because of the gating of voltage-dependent (Na^+) channels [see F. T. Horrigan and R. J. Bookman, *Neuron* **13**, 1119 (1994)] because (i) inhibition of the voltage-gated Ca^{2+} channels with Co^{2+} abolishes depolarization-evoked capacitance changes [C. Ämmälä *et al.*, *J. Physiol. (London)* **474**, 665 (1993)] and (ii) lowering the temperature from +34°C to room temperature suppresses exocytosis (and capacitance increases) while not much affecting the properties of the voltage-gated currents (E. Renström, L. Eliasson, P. Rorsman, in preparation).
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8. In the simultaneous presence of both 17 mM glucose and 40 mM K^+, the sulfonylurea glibenclamide (2 μM) increased insulin secretion by 210 ± 70% (from 1.50 ± 0.19 ng per 20 islets per 10 min to 4.24 ± 0.87 ng per 20 islets per 10 min; $P < 0.01$ versus the value observed in the presence of glucose and K^+ alone). Experiments were performed at 37°C. Values are means ± SEM of nine experiments carried out on three preparations of islets.
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11. $[Ca^{2+}]_i$ was measured in parallel with the electrophysiological recordings by dual wavelength microfluorimetry as described (17) with the use of fura-2 as the indicator. Before the measurements, the cells were loaded with the ester form (0.1 μM) of fura for 20 min.
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18. The phase angle was determined for each experiment. The absence of a change in cell conductance associated with the depolarizations indicates that the phase angle was correctly set and that the observed step changes of membrane capacitance are not attributable to variations of cell conductance.
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Cold-Induced Expression of Δ^9-Desaturase in Carp by Transcriptional and Posttranslational Mechanisms

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Poikilothermic animals respond to chronic cold by increasing phosphoglyceride unsaturation to restore the fluidity of cold-rigidified membranes. Despite the importance of this compensatory response, the enzymes involved have not been clearly identified, and the mechanisms that control their activity are unknown. In carp liver, cold induces an 8- to 10-fold increase in specific activity of the microsomal stearoyl coenzyme A desaturase. Cold-induced up-regulation of gene transcription resulted in a 10-fold increase in desaturase transcript amounts after 48 to 60 hours. However, this increase was preceded by the activation of latent desaturase, probably by a posttranslational mechanism. These two mechanisms may act sequentially to match desaturase expression to the demands imposed by a progressive decrease in temperature.

Cold is a major environmental problem for all living organisms. Poikilothermic animals respond adaptively to chronic cold by a suite of cellular responses that compensate to varying extents for the rate-depressing effects of cooling. The most widespread and consistent response is to increase the unsaturation of membrane phospholipids to offset a cold-induced rigidification of bilayer lipids (1, 2). This "homeoviscous adaptation" is an example of the cell membrane regulation displayed by all cells, yet the enzymes involved have not been clearly identified, and the mechanisms that control their activity are unknown. Cooling of carp liver causes a large increase in the activity of the Δ^9-desaturase (3), the enzyme that incorporates the first unsaturation bond into saturated fatty acids (4). Here, we investigated the mechanisms underlying this response by measuring amounts of desaturase transcript and protein.

Carp that had been maintained at 30°C for at least 3 months were cooled over 3 days to 10°C and held at 10°C (5). At intervals, the carp were killed and liver microsomes were prepared. Figure 1 shows changes in the fatty acid composition of the

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