Sulfonylureas Enhance Exocytosis From Pancreatic β-Cells by a Mechanism That Does Not Involve Direct Activation of Protein Kinase C

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Hypoglycemic sulfonylureas stimulate insulin release by binding to a regulatory subunit of plasma membrane ATP-sensitive K^+ (K_{ATP}) channels. The consequent closure of K_{ATP} channels leads to depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and a rise in intracellular [Ca^{2+}]. Recently, however, it has been suggested that sulfonylureas may have an additional action on secretion, independent of changes in intracellular [Ca^{2+}] but dependent on the activity of protein kinase C (PKC). We have investigated the mechanisms involved in the PKC-dependent effect of sulfonylureas on the secretion machinery in β-cells. In MIN6 β-cells permeabilized by streptolysin O, insulin release was stimulated by elevation of [Ca^{2+}] from 10^{-8} to 10^{-5} mol/l. At a [Ca^{2+}] of 10^{-8} mol/l, insulin release from permeabilized β-cells was stimulated by addition of GTP-γ-S, or by addition of a phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). TPA, but not GTP-γ-S, also increased insulin release when [Ca^{2+}] was 10^{-5} mol/l. Insulin release from permeabilized β-cells was stimulated by tolbutamide (0.1–1 mmol/l) at 10^{-8} but not at 10^{-5} mol/l Ca^{2+}. The effect of tolbutamide was blocked either by inhibition of PKC or when phorbol ester–sensitive PKC isoforms were maximally stimulated by TPA. Meglitinide and glibenclamide also stimulated insulin release from permeabilized β-cells. To assess the possibility that direct activation of PKC mediates the exocytotic response to sulfonylureas, we studied the effect of tolbutamide and glibenclamide on PKC activity. Purified brain PKC was not activated by tolbutamide or glibenclamide, whether tested in the absence or presence of phosphatidylserine or TPA, or at low or high [Ca^{2+}]; nor was the total PKC activity in extracts of MIN6 β-cells affected by tolbutamide. Neither tolbutamide nor glibenclamide elicited translocation of any isoform of PKC in intact or permeabilized β-cells under conditions in which TPA evoked a marked redistribution of PKC α- and ε-isoforms. We conclude that although the plasma membrane K_{ATP} channel–independent stimulation of exocytosis by sulfonylureas may require functional PKC, the mechanism does not involve a direct activation of the enzyme.
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BSA, bovine serum albumin; DAG, diacylglycerol; K_{ATP} channel, ATP-sensitive K^+ channel; PBS, phosphate-buffered saline; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Sulfonylureas such as tolbutamide and glibenclamide stimulate insulin secretion from pancreatic β-cells principally by inhibiting ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane (1). This inhibition leads to membrane depolarization, activation of voltage-gated Ca^{2+} channels, increased Ca^{2+} influx, a rise in cytosolic [Ca^{2+}], and thereby insulin release. In addition to their action on K_{ATP} channels, sulfonylureas have also been reported to stimulate exocytosis directly, by a mechanism that is independent of plasma membrane depolarization (2). This effect, measured using the capacitance technique, was abolished by inhibitors of protein kinase C (PKC) or when exocytosis was potentiated by stimulation of phorbol ester–sensitive PKC isoforms. It was concluded that sulfonylureas have a direct effect on β-cell exocytosis, and that the effect is mediated by a pathway involving PKC activation. These findings have been challenged, however, because no evidence could be obtained for the direct effect of sulfonylureas on insulin release from intact mouse islets (3). In the present study, we have used permeabilized β-cells to reexamine the direct effect of sulfonylureas on exocytosis.

There is evidence in other tissues of direct stimulation of PKC by sulfonylureas (4,5). Because such a mechanism could provide a simple explanation for the K_{ATP} channel–independent action of sulfonylureas on exocytosis, we have also examined the ability of tolbutamide and glibenclamide to activate β-cell PKC.

RESEARCH DESIGN AND METHODS

Materials. All tissue culture reagents were obtained from Life Technologies (Paisley, Scotland, U.K.) or Sigma (Poole, England, U.K.). Tolbutamide was obtained from Sigma, and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was obtained from Alexis (Nottingham, U.K.). The PKC Assay Kit was obtained from Promega (Southampton, U.K.). Ro 31-8220 was a gift from D. Bradshaw (Roche Research Centre, Welwyn Garden City, Herts, U.K.). Streptolysin O was supplied by Murex (Paris). Purified rat brain PKC was obtained from Calbiochem (Nottingham, U.K.). Primary antibodies anti–PKC-α, -λ, and -μ were obtained from Transduction Laboratories (Affiniti) (Exeter, U.K.); anti–PKC-βII was obtained from Gibco (Paisley, Scotland, U.K.); and anti–PKC-ε and -ζ were provided by P.J. Parker (Imperial Cancer Research Fund, London). Anti-actin primary antibodies and alkaline phosphatase–conjugated secondary antibodies were obtained from Sigma. Solid-phase anti–guinea pig serum (Sac-Cel) was obtained from IDS (Boldon, Tyne and Wear, U.K.). ^{125}I-labeled insulin was obtained from Du Pont-NEN (Hounslow, U.K.). [γ-^{32}P]ATP was obtained from Amersham (Little Chalfont, Bucks, U.K.). Other chemicals, of reagent grade, were obtained from Sigma or BDH-Merck (Lutterworth, Leics, U.K.).

Cell culture. MIN6 β-cells were cultured as previously described (6). For measurement of insulin secretion from permeabilized cells, the cells were seeded in Multiwells at an initial cell density of 0.2×10^6 cells per milliliter and were cultured for 1–2 days before use.

TABLE 1
Insulin release from permeabilized β-cells

Line	Additions	Insulin release (μU · well^{-1} · [20 min]$^{-1}$) 10^{-8} mol/l [Ca^{2+}]	10^{-5} mol/l [Ca^{2+}]
1	None (control)	651 ± 44 (32)	1,531 ± 113 (32)*
2	10 μmol/l GTP-γ-S	1,696 ± 93 (8)†	2,053 ± 197 (8)‡¶
3	0.1 μmol/l TPA	2,643 ± 98 (8)†	2,768 ± 216 (8)†‡
4	0.1 μmol/l TPA + 10 μmol/l Ro 31-8220	759 ± 50 (8)§	1,635 ± 103 (8)*§
5	1 mmol/l tolbutamide	1,347 ± 120 (24)†	1,811 ± 178 (24)‖¶
6	1 mmol/l tolbutamide + 0.1 μmol/l TPA	2,413 ± 189 (8)#	2,823 ± 173 (8)‡#
7	1 mmol/l tolbutamide + 10 μmol/l Ro 31-8220	896 ± 195 (16)**	1,118 ± 158 (16)‡ ††
8	10 μmol/l Ro 31-8220	660 ± 48 (4)¶	1,644 ± 241 (4)*¶

Data are means ± SE (n of observations). Insulin release from permeabilized MIN6 β-cells was measured (as described in METHODS) in the presence of the agents indicated and with a free [Ca^{2+}] of 10^{-8} or 10^{-5} mol/l, as indicated. *$P < 0.001$ vs. [Ca^{2+}] of 10^{-8} mol/l with the same addition; †$P < 0.001$ vs. line 1 at the same [Ca^{2+}]; ‡NS vs. [Ca^{2+}] of 10^{-8} mol/l with the same addition; §$P < 0.001$ vs. line 3 at the same [Ca^{2+}]; ‖$P < 0.05$ vs. [Ca^{2+}] of 10^{-8} mol/l with the same addition; ¶NS vs. line 1 at the same [Ca^{2+}]; #NS vs. line 3 at the same [Ca^{2+}]; **$P < 0.05$, ††$P < 0.01$ vs. line 5 at the same [Ca^{2+}].

Insulin secretion from permeabilized β-cells. Tissue culture medium was removed, and the cells were preincubated for 1 h in HEPES-buffered bicarbonate medium at 37°C. The medium was removed, and the cells were washed twice in buffer A (16 mmol/l HEPES [pH 7], 100 mmol/l potassium glutamate, 42 mmol/l sodium glutamate, 3 mmol/l MgATP, 5 mg/ml bovine serum albumin [BSA], and 1 mmol/l EGTA). The cells were permeabilized by the addition of 0.5 U reduced streptolysin O in 0.5 ml of buffer A containing test agents.The free [Ca^{2+}] of the medium was adjusted by the addition of $CaCl_2$. Incubation was continued for 20 min at 37°C. Samples of medium were removed and assayed for insulin by radioimmunoassay as previously described (7).

PKC assay. PKC enzyme activity was assayed by measuring the incorporation of ^{32}P into biotinylated peptide neurogranin$_{28-43}$ using the Promega PKC Assay System. The reaction medium contained 20 mmol/l Tris/HCl (pH 7.5), 10 mmol/l $MgCl_2$, 0.1 mmol/l peptide substrate, 32 μg/ml phosphatidylserine (PS), 0.35 μg/ml purified rat brain PKC or 1.3 mg/ml MIN6 cell extract (see below), and 0.1 mmol/l [γ-^{32}P]ATP (100–200 cpm/pmol). EGTA (0.25 mmol/l) and a calculated amount of $CaCl_2$ were added to give a free [Ca^{2+}] of either 200 nmol/l or 150 μmol/l. The reaction was started by adding ATP and was carried out at 30°C for 6 min in a total volume of 50 μl. The reaction was terminated by adding 25 μl of 7.5 mol/l guanidine HCl, and then 15–20 μl of the mixture was spotted onto a streptavidin disk and washed 10 times as specified by the manufacturer. The disk was rinsed briefly in water and air-dried for 30 min, and the radioactivity was counted in 10 ml of scintillation fluid.

Preparation of cell extract for PKC assay. Confluent MIN6 cells were trypsinized and washed twice in phosphate-buffered saline (PBS). The cells were resuspended in an ice-cold lysis buffer (25 mmol/l Tris/HCl [pH 7.4], 0.5 mmol/l EGTA, 0.5 mmol/l EDTA, 1% Triton X-100, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l benzamidine, 2 μg/ml aprotinin, 10 μg/ml pepstatin, and 2 μg/ml leupeptin) and were incubated on ice for 30 min with occasional mixing. The mixture was centrifuged at 16,000g at 4°C for 5 min, and the supernatant (10 mg/ml) was used for PKC assay. The protein content of the cell extract was determined (8), using BSA as a standard.

PKC translocation. For measurement of PKC translocation in permeabilized cells, the cells were permeabilized and incubated as described above for insulin secretion. The medium was removed and the cells were scraped into ice-cold buffer B (20 mmol/l Tris/HCl [pH 7.4], 10 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l dithiothreitol, 1 mmol/l PMSF, 1 mmol/l benzamidine, 2 μg/ml aprotinin, 10 μg/ml pepstatin, and 2 μg/ml leupeptin). For measurement of PKC translocation in intact cells, confluent MIN6 cells were trypsinized and washed twice in PBS. The cells were resuspended in HEPES-buffered bicarbonate medium, containing 20 mmol/l HEPES/NaOH (pH 7.4), 119 mmol/l NaCl, 4.75 mmol/l KCl, 5.0 mmol/l $NaHCO_3$, 2.54 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 0.5 mmol/l glucose, and 0.2% BSA, and then the cells were preincubated at 37°C for 1 h. The cells were divided into equal aliquots and incubated in the above-mentioned medium plus test agent at 5×10^6 cells per milliliter at 37°C for 30 min. The cell pellet was resuspended in ice-cold buffer B.

Cell lysates were prepared by three immediate freeze/thaw cycles and were centrifuged at 100,000g for 1 h at 4°C; the supernatant was collected as the cytosol fraction. The pellet was resuspended in buffer (50 mmol/l Tris/HCl [pH 7.4], 150 mmol/l NaCl, 1 mmol/l EGTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/l Na_3VO_4, 1 mmol/l NaF, 1 mmol/l dithiothreitol, 1 mmol/l PMSF, 1 mmol/l benzamidine, 2 μg/ml aprotinin, 10 μg/ml pepstatin and 2 μg/ml leupeptin) and incubated at 4°C for 30 min with occasional mixing. The mixture was centrifuged at 16,000g for 5 min at 4°C, and the supernatant was taken as the membrane fraction.

The two fractions of the cell extract were analyzed for PKC isoforms by immunoblotting using isoform-specific anti-PKC antibodies as described previously (6).

Solutions. Glibenclamide, Ro 31-8220, and TPA were prepared as stock solutions in DMSO. Tolbutamide was prepared as a stock solution either in DMSO or by addition of an equimolar amount of KOH. An aliquot of PS in chloroform (10 mg/ml) was spin-dried under vacuum at room temperature and resuspended in ice-cold 20 mmol/l Tris/HCl (pH 7.4) at 800 μg/ml by sonication on ice for 5 min. This stock was stored at 4°C and used within 2 months. The suspension was vortexed for 20 s just before use in the PKC assay.

Data analysis. Data are expressed as means ± SE. Statistical significance was evaluated using Student's t test.

RESULTS

Insulin secretion from permeabilized β-cells. The data in Table 1 show that insulin secretion from MIN6 β-cells permeabilized by streptolysin O was increased 2.5-fold on raising the [Ca^{2+}] from 10^{-8} to 10^{-5} mol/l; at a [Ca^{2+}] of 10^{-8} mol/l, but not at 10^{-5} mol/l, secretion was stimulated twofold by 1 μmol/l GTP-γ-S. The effects of tolbutamide and of modifiers of PKC activity are also shown in Table 1. Activation of PKC by 0.1 μmol/l TPA caused a marked (fourfold) enhancement of insulin release at a [Ca^{2+}] of 10^{-8} mol/l and a smaller (1.8-fold) increase at a [Ca^{2+}] of 10^{-5} mol/l. In the presence of 0.1 μmol/l TPA, insulin release at 10^{-5} mol/l was no greater than that at 10^{-8} mol/l, consistent with TPA increasing the sensitivity of PKC to Ca^{2+}. The secretion elicited by TPA was markedly reduced by Ro 31-8220, an inhibitor of PKC. However, Ro 31-8220 itself did not inhibit insulin release in the absence of other additions. Tolbutamide (1 mmol/l) increased insulin release twofold at a [Ca^{2+}] of 10^{-8} mol/l but did not further augment the secretion elicited by a [Ca^{2+}] of 10^{-5} mol/l. Ro 31-8220 inhibited secretion stimulated by tolbutamide. Bis-indolyl maleimide (10 μmol/l), another PKC inhibitor, also significantly reduced tolbutamide-stimulated insulin release to 69 ± 4% of its value in the absence of the inhibitor ($n = 8$; $P < 0.05$). The effects of tolbutamide and TPA were not additive—secretion in the combined presence of TPA and tolbutamide was not greater than that in the presence of TPA alone. Insulin release at a [Ca^{2+}] of 10^{-8} mol/l was also increased to 192 ± 12% of control ($n = 4$) by 1 mmol/l meglitinide and to 163 ± 7% of control ($n = 8$) by 1 μmol/l glibenclamide. As shown in Fig. 1, at a

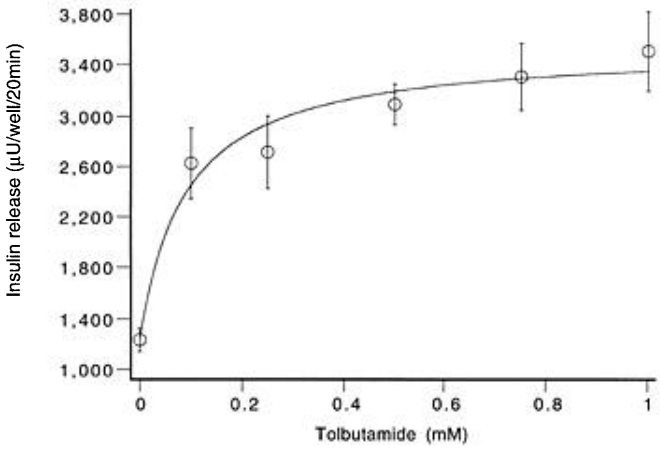

FIG. 1. Concentration dependence of tolbutamide stimulation of insulin release from permeabilized MIN6 β-cells. Insulin release was measured (as described in METHODS) at the concentrations of tolbutamide indicated and at a free [Ca^{2+}] of 10^{-8} mol/l. Rates of insulin release are shown as means ± SE (n = 8).

[Ca^{2+}] of 10^{-8} mol/l, tolbutamide was effective in eliciting insulin release over the range of 0.1–1 mmol/l.

Effects of sulfonylureas on PKC activity. At a [Ca^{2+}] of 150 µmol/l, PS stimulated purified brain PKC (mainly α-, β-, and γ-isoforms) 8.53-fold. As shown in Table 2, addition of 1 µmol/l TPA in the presence of PS produced a further stimulation of 1.95-fold. However, neither 1 mmol/l tolbutamide nor 1 µmol/l glibenclamide had any significant stimulatory effect. Because it was possible that in the cellular environment, sulfonylureas may act synergistically with diacylglycerol (DAG) in the membrane rather than substituting for it, we also tested the ability of tolbutamide to activate PKC in the presence of a submaximally stimulating TPA concentration. Neither tolbutamide nor glibenclamide increased the activity of PKC in the presence of 0.1 µmol/l TPA, which itself elicited a 1.52-fold stimulation. At a lower [Ca^{2+}] (0.2 µmol/l), stimulation with PS alone was lower (1.37-fold), and the further stimulation through addition of 1 µmol/l TPA was higher (5.15-fold), as expected. Neither tolbutamide (1 mmol/l) nor glibenclamide (1 µmol/l) stimulated PKC at a [Ca^{+2}] of 0.2 µmol/l. In the absence of PS, no stimulation of PKC by either sulfonylurea was observed (data not shown). Thus, sulfonylureas neither mimic nor synergize with the effect of TPA on PKC.

Using a MIN6 cell extract containing PKC-ε, -ζ, -λ, and -µ in addition to α- and βII-isoforms (6), similar data were obtained. Thus, at a [Ca^{2+}] of 150 µmol/l, neither 1 mmol/l tolbutamide nor 1 µmol/l glibenclamide reproduced the stimulatory effect (3.3-fold) on PKC activity of 1 µmol/l TPA in the presence of PS. Nor was the stimulation of PKC by a submaximally effective concentration of TPA (0.1 µmol/l) augmented by tolbutamide or glibenclamide. Tolbutamide was also ineffective in the absence of PS (109 ± 3% of control; n = 3). Tolbutamide also failed to stimulate PKC activ-

TABLE 2
Effects of TPA and sulfonylureas on PKC activity

PKC	[Ca^{2+}] (µmol/l)	Addition	PKC activity (% control)
Brain (purified)	150	None (control)	100
		1 µmol/l TPA	195 ± 9 (5)*
		1 mmol/l tolbutamide	87 ± 5 (6)
		1 µmol/l glibenclamide	113 ± 8 (4)
		0.1 µmol/l TPA	152 ± 12 (9)*
		0.1 µmol/l TPA + 1 mmol/l tolbutamide	150 ± 11 (5)*†
		0.1 µmol/l TPA + 1 µmol/l glibenclamide	154 ± 8 (5)*†
	0.2	None (control)	100
		1 µmol/l TPA	515 ± 11*
		1 mmol/l tolbutamide	108 ± 0.2
		1 µmol/l glibenclamide	95 ± 2
MIN6 β-cell extract	150	None (control)	100
		1 µmol/l TPA	327 ± 51‡
		1 mmol/l tolbutamide	89 ± 6
		1 µmol/l glibenclamide	90 ± 7
		0.1 µmol/l TPA	159 ± 12§
		0.1 µmol/l TPA + 1 mmol/l tolbutamide	153 ± 21†‡
		0.1 µmol/l TPA + 1 µmol/l glibenclamide	153 ± 11†§
	0.2	None (control)	100
		1 µmol/l TPA	231 ± 3*
		1 mmol/l tolbutamide	103 ± 5

Data are means ± SE, expressed as percentage of control rate in the absence of additions. Values in parentheses indicate number of experiments (n = 3 where not indicated). PKC assays were carried out at 30°C for 6 min with 0.35 µg/ml purified rat brain PKC or 1.3 mg/ml MIN6 β-cell extract in the presence of 23 µg/ml PS with the free [Ca^{2+}] and additions indicated. The mean stimulation of brain PKC elicited by PS was 8.53- and 1.36-fold at a [Ca^{2+}] of 150 and 0.2 µmol/l, respectively. Increased [Ca^{2+}] did not increase PKC activity in the absence of PS but caused a mean 3.84-fold increase in the presence of PS. *P < 0.001 vs. control; †NS vs. 0.1 µmol/l TPA; ‡P < 0.05, §P < 0.01 vs. control.

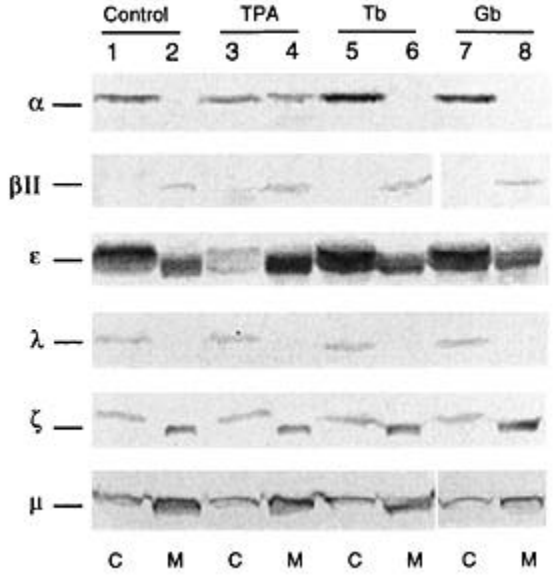

FIG. 2. The effect of TPA and sulfonylureas on the subcellular distribution of PKC isoforms in permeabilized MIN6 β-cells. MIN6 β-cells were preincubated at 37°C for 1 h and were then permeabilized with streptolysin O and incubated for 20 min in the absence (*lanes 1* and *2*) or presence of 0.1 μmol/l TPA (*lanes 3* and *4*), 1 mmol/l tolbutamide (Tb) (*lanes 5* and *6*), or 1 μmol/l glibenclamide (Gb) (*lanes 7* and *8*). Membrane (M) (*lanes 1, 3, 5,* and *7*) and cytosolic (C) (*lanes 2, 4, 6,* and *8*) fractions were prepared as described in METHODS. The fractions were subjected to SDS-PAGE, and the levels of the PKC isoforms indicated were analyzed by Western blotting.

ity in the MIN6 cell extracts at the lower [Ca^{2+}] in contrast to the 2.3-fold stimulation elicited by TPA.

Effects of sulfonylureas on translocation of PKC in β-cells. Permeabilized MIN6 cells were incubated with either TPA or sulfonylureas and then fractionated into cytosol and membrane fractions. Both fractions were then subjected to SDS-PAGE, and the level of PKC isoforms was analyzed by immunoblotting using isoform-specific anti-PKC antibodies. As shown in Fig. 2, both PKC-α and -ε of permeabilized MIN6 cells were translocated from cytosol to membrane on incubation with 0.1 μmol/l TPA at 37° for 20 min. In contrast, tolbutamide (1 mmol/l) or glibenclamide (1 μmol/l) did not cause translocation of these PKC isoforms under the same conditions. PKC-βII and -μ were associated with the membrane fraction under control conditions, and no change in distribution was elicited by TPA or by sulfonylureas. The distribution of PKC-λ and -ζ between membrane and cytosol was not affected by TPA or sulfonylureas. In intact MIN6 β-cells, TPA similarly elicited translocation of PKC-α and -ε, but 1 mmol/l tolbutamide was ineffective (data not shown).

DISCUSSION

Permeabilized β-cells and islets of Langerhans have been widely used to study the mechanism of exocytosis (9–16). However, the technique has not previously been used for MIN6 β-cells; therefore, it was first necessary to establish suitable conditions for obtaining Ca^{2+}-dependent secretion of insulin. We chose to use streptolysin O for permeabilization because *1*) it has been demonstrated in mammalian cells that streptolysin O does not permeabilize internal membrane compartments (17), and *2*) it has been found in pancreatic islets treated with streptolysin O that although the holes formed are large enough to permit entry of antibodies, egress of cytosolic constituents is restricted—thus, leakage of myosin light chain kinase (140 kDa) and of myosin light chain (18 kDa) was only minor during a 45-min period (18). Using streptolysin O to permeabilize MIN6 β-cells under the conditions described in METHODS, we found that insulin secretion could be stimulated by increasing [Ca^{2+}] from 10^{-8} to 10^{-5} mol/l or by addition of GTP-γ-S. Stimulation under these conditions has generally been found to be characteristic of exocytosis from permeabilized cells (16,19–21). Thus, permeabilized MIN6 β-cells display the expected properties for study of exocytosis.

In agreement with data for other permeabilized insulin-secreting cells (11,22,23), TPA markedly enhanced insulin release from permeabilized MIN6 β-cells. The effect is attributable to activation of PKC because *1*) it was blocked by an inhibitor of PKC, and *2*) secretion in the presence of TPA no longer depended on a raised [Ca^{2+}] level. Consistent with previous studies using the patch clamp technique (2), our secretion data in permeabilized β-cells indicate that sulfonylureas can elicit insulin secretion under conditions in which the mechanism cannot involve changes in intracellular [Ca^{2+}] and is therefore independent of K_{ATP} channel activity. The data also provide some support for the idea that the plasma membrane K_{ATP} channel–independent potentiation of insulin release by sulfonylureas may require the functional activity of PKC. Thus, the effect of tolbutamide on exocytosis was reduced by the presence of PKC inhibitors and was not evident when PKC was maximally stimulated by TPA. It seems likely that activity of either PKC-α or PKC-ε is required for the effect of sulfonylureas on exocytosis because only these two isoforms are activated by TPA in MIN6 β-cells (6).

These data and those previously reported (2) could be most easily explained by a direct action of sulfonylureas on PKC. In other tissues, such a stimulatory effect of sulfonylureas on PKC has indeed been observed (4,5). However, our data do not support this hypothesis. At a concentration that elicited insulin secretion from permeabilized β-cells, tolbutamide had no effect on the activity of purified rat brain PKC (mainly PKC-α, -β and -γ), whether tested in the absence or presence of stimulatory concentrations of Ca^{2+}, PS, or TPA. Furthermore, glibenclamide did not affect PKC activity, nor could we detect any effect of tolbutamide on PKC activity in MIN6 β-cell homogenates, which we have shown to contain, in addition, βII-, ε-, λ-, μ- and ζ-isoforms (6).

It was still possible that in the cellular environment, sulfonylureas could be activating PKC directly. As an index of PKC activation in cells, we measured the translocation of specific PKC isoforms from a cytosolic to a membrane location. The results were the same whether these experiments were conducted on permeabilized β-cells under conditions identical to those used for measurement of insulin secretion or on intact β-cells. TPA caused the translocation of PKC-α and -ε. The failure of TPA to cause translocation of PKC-ζ and -λ is consistent with the insensitivity of PKC-ζ and -λ to DAG and phorbol esters. The translocation of PKC-βII and -μ could not be assessed because these isoforms were distributed predominantly in the membrane fraction under control conditions. However, the possibility of translocation of these forms is small because we have previously shown that they are insensitive to downregulation by TPA in the β-cell

(6). If sulfonylureas mimic TPA, then we should detect a similar pattern of translocation of PKC with tolbutamide as with TPA under our conditions. This pattern was not observed because tolbutamide and glibenclamide did not elicit translocation of any of these isoforms. However, there is evidence that PKC that is targeted to membranes after activation by Ca^{2+} may in fact redistribute to the soluble fraction on extraction under conditions of low [Ca^{2+}] (24). Therefore, if tolbutamide were to interact in some way with Ca^{2+}, then we might miss the evoked translocation under our extraction conditions. Although this possibility cannot be entirely ruled out, it is unlikely because the [Ca^{2+}] is fixed at a very low level in the permeabilized cells and cannot be changed by tolbutamide; it is not obvious how tolbutamide could mimic Ca^{2+}-evoked translocation.

Thus, sulfonylureas have no measurable effect on PKC translocation in permeabilized or intact β-cells, on the activity of purified rat brain PKC, or on PKC activity in MIN6 β-cell homogenates. These data indicate that sulfonylureas do not directly stimulate β-cell PKC activity, in contrast to findings reported for adipocytes (4) and smooth muscle cells (5). We conclude that although functional PKC may be required for plasma membrane K_{ATP} channel–independent stimulation of insulin release by sulfonylureas, the mechanism does not involve direct activation of the enzyme.

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