Enhanced Stimulus–Secretion Coupling in Polyamine-depleted Rat Insulinoma Cells

An Effect Involving Increased Cytoplasmic Ca^{2+}, Inositol Phosphate Generation, and Phorbol Ester Sensitivity

Åke Sjöholm,*‡§ Per Arkhammar,* Nils Welsh,‡ Krister Bokvist,|| Patrik Rorsman,|| Anders Hallberg,‡ Thomas Nilsson,* Michael Welsh,‡ and Per-Olof Berggren*

**Department of Endocrinology, The Rolf Luft Center for Diabetes Research, Karolinska Institute, Karolinska Hospital, S-104 01 Stockholm, Sweden; ‡Department of Medical Cell Biology, Uppsala University, Biomedical Center, S-751 23 Uppsala, Sweden; §Department of Internal Medicine, Löwenströmska District County Hospital, S-194 03 Upplands Väsby, Sweden; and ||Department of Medical Physics, University of Gothenburg, S-400 33 Gothenburg, Sweden*

Abstract

To extend previous observations on the role of polyamines in insulin production, metabolism, and replication of insulin-secreting pancreatic β cells, we have studied the role of polyamines in the regulation of the stimulus-secretion coupling of clonal rat insulinoma cells (RINm5F). For this purpose, RINm5F cells were partially depleted in their polyamine contents by use of the specific ornithine decarboxylase inhibitor difluoromethylornithine (DFMO), which led to an increase in cellular insulin and ATP contents. Analysis of different parts of the signal transduction pathway revealed that insulin secretion and the increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) after K^+-induced depolarization were markedly enhanced in DFMO-treated cells. These effects were paralleled by increased voltage-activated Ca^{2+} currents, as judged by whole-cell patch-clamp analysis, probably reflecting increased channel activity rather than elevated number of channels per cell. DFMO treatment also rendered phospholipase C in these cells more sensitive to the muscarinic receptor agonist carbamylcholine, as evidenced by enhanced generation of inositol phosphates, increase in $[Ca^{2+}]_i$ and insulin secretion, despite an unaltered ligand binding to muscarinic receptors and lack of effect on protein kinase C activity. In addition, the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate, at concentrations suggested to be specific for protein kinase C activation, evoked an increased insulin output in polyamine-deprived cells compared to control cells. The stimulatory effects of glucose or the cyclic AMP raising agent theophylline on insulin release were not increased by DFMO treatment. In spite of increased binding of sulfonylurea in DFMO-treated cells, there was no secretory response or altered increase in $[Ca^{2+}]_i$ in response to the drug in these cells. It is concluded that partial polyamine depletion sensitizes the stimulus-secretion coupling at multiple levels in the insulinoma cells, including increased voltage-dependent Ca^{2+} influx and enhanced responsiveness to activators of phospholipase C and protein kinase C. In their entirety, our present results indicate that the behavior of the stimulus-secretion coupling of polyamine-depleted RINm5F insulinoma cells changes towards that of native β cells, thus improving the usefulness of this cell line for studies of β cell insulin secretion. (*J. Clin. Invest.* 1993. 92:1910–1917.) Key words: insulin secretion • polyamines • protein kinase C • inositol trisphosphate • Ca^{2+} channels

Introduction

The polyamines putrescine, spermidine, and spermine are aliphatic cations controlling the proliferative activity and differentiation state in many cell systems (1). High concentrations of polyamines have been reported to occur in both endocrine and exocrine pancreatic cells (2). Among the pancreatic cell populations, the insulin-producing β cell in particular displays conspicuously high polyamine concentrations (3), a feature shared by certain other endocrine cells (4). In normal pancreatic β cells, we have previously reported that polyamines act as permissive or stimulatory factors in insulin production and secretion (5–13). Other investigators, however, suggested an inhibitory role for polyamines in insulin secretion (14) when these substances were directly added to β cells.

The aim of this paper was to study the role of polyamines in clonal rat insulinoma cells (RINm5F).[1] To this end, partial depletion of the cellular polyamine content was achieved by addition of the specific and irreversible ornithine decarboxylase inhibitor difluoromethylornithine (DFMO) (15). Initial findings indicated that RINm5F cells treated with DFMO markedly decreased their proliferative activity and displayed an increased content of insulin, insulin secretory granules and ATP, along with changes in the aberrant substrate metabolism of the tumoral cells towards that of normal pancreatic β cells (16). We thus aimed at elucidating whether there was also an enhanced insulin output from the DFMO-treated cells in response to different stimuli and, if so, by which mechanisms this is achieved. For this purpose, RINm5F cells were cultured in the absence or presence of DFMO, and the impact of various

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Address correspondence and reprint requests to Åke Sjöholm, Department of Endocrinology, The Rolf Luft Center for Diabetes Research, Karolinska Institute, Karolinska Hospital, Box 60 500, S-104 01, Stockholm, Sweden.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; DFMO, difluoromethylornithine; $InsP_3$, inositol trisphosphate; RINm5F, clonal rat insulinoma cells; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

secretagogues on different parts of the stimulus-secretion coupling was monitored.

Methods

Materials. [2-^{3}H]Inositol (20 Ci/mmol), [^{3}H]*N*-methyl-scopolamine (83 Ci/mmol), ^{32}P (3,000 Ci/mmol), and Megaprime were from Amersham International (Bucks, United Kingdom). Protein A–Sepharose was obtained from Pharmacia (Uppsala, Sweden). Carbamylcholine, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), tetrodotoxin, putrescine, spermidine, spermine, L-proline, *N*-methylscopolamine, and quin 2/acetoxymethylester were from Sigma Immunochemicals (St. Louis, MO). Theophylline was from Apoteksbolaget (Stockholm, Sweden) and D-600 was a generous gift from Knoll A.G. (Ludwigshafen am Rhein, Germany). Tolbutamide, [^{3}H]-glibenclamide, and unlabeled glibenclamide were kindly given by Hoechst A.G. (Frankfurt, Germany). Bisoxonol was from Molecular Probes (Junction City, OR); and diazoxide was a gift from Schering Corp., (Kenilworth, NJ). ATP standard was from Boehringer-Mannheim (Mannheim, Germany). Culture medium RPMI 1640, fetal calf serum, L-glutamine, calcium-, and magnesium-free Hanks' balanced salt solution, bensylpenicillin, and streptomycin were from Flow Laboratories, (Irvine, United Kingdom). Antibovine insulin serum was supplied by Miles-Yeda (Rehovot, Israel). Crystalline mouse insulin and ^{125}I-labeled insulin were from Novo (Copenhagen, Denmark). Trypsin (0.05%)/EDTA (0.02%) solution was supplied by Gibco (Paisley, United Kingdom). The fast-track kit was provided by Invitrogen (San Diego, CA).

Cell culture. RINm5F cells were cultured for 4 d in medium RPMI 1640 and 10% fetal calf serum with or without 1 mM DFMO. During this period, cells were in logharithmic growth phase (16). Cell viability was assessed by the ability to exclude Trypan blue (0.2%).

Polyamine content. The cellular polyamine content was determined by one-dimensional thin layer chromatography (16, 17). For this purpose, groups of 3–4 $\times$ 10^5 cells were scraped off the culture dishes, washed in PBS, and homogenized by sonication in 20 μl 0.3 M perchloric acid at 4°C. After centrifugation (5 min; 12,000 *g*), the supernatant was alkalinized by addition of 10 μl 1 M Na_2CO_3 followed by 75 μl of 10 mg/ml dansyl chloride in acetone. After incubation at room temperature overnight in the dark, excess reagent was reacted with 5 μl L-proline (250 mg/ml) followed by sonication for 2 min. Dansyl-polyamines were extracted in 100 μl toluene. The toluene was evaporated and the residue redissolved in 5 μl toluene for application on thin layer chromatography plates (HPTLC Fertigplatten, Kieselgel 60 F_{254}; E. Merck, Darmstadt, Germany). The dansylated polyamines were separated by one run with ethyl acetate/cyclohexane (1:1, vol/vol) followed by two runs with diethyl ether/cyclohexane (2:3, vol/vol). The spots were scraped off the plates and the fluorescence intensities of the supernatants measured in a luminescence spectrometer (model LS5; Perkin-Elmer Corp., Norwalk, CT) connected to a plate reader at an excitation wavelength of 360 nm and an emission wavelength of 510 nm. This method has, in our hands, an intra-assay variability (SEM/mean) of 6%, an interassay variability of 3%, and a sensitivity of $\sim$ 15 pmol (i.e., an amount of polyamine resulting in a fluorescence intensity two standard deviations above the blank reading). Standard curves were linear up to $\geq$ 1,000 pmol and showed correlation coefficients of $\geq$ 0.99.

Insulin secretion and contents of insulin, ATP, and DNA. On day 4 of culture, media were removed and the cultures were washed once with phosphate-buffered saline. They were then preincubated at 37°C for 45 min in Krebs-Ringer bicarbonate buffer (18) at pH 7.4 containing 2 mg/ml bovine serum albumin, 10 mM Hepes, and 2.8 mM glucose. The preincubation media were then discarded, the cultures were washed once with similar media and subsequently exposed for 30 min to different secretagogues in similar incubation buffer with 2.8 mM glucose. Aliquots of the centrifuged incubation media were analyzed radioimmunologically for insulin (19). The amount of insulin in acid ethanol extracts of cell homogenates (16) was analyzed radioimmunologically (19).

For determination of the cellular ATP content, cell cultures were quickly washed once with ice-cold PBS and directly sonicated in ice-cold 0.89 M perchloric acid and 2 mM EDTA, and immediately plunged into liquid nitrogen and kept frozen at −80°C pending analysis. ATP was determined by use of a bioluminescence kit (5, 16). Light signals were quantified at room temperature in a luminometer (model 1250; LKB Instruments, Bromma, Sweden) connected to a voltmeter.

For DNA measurements, cells were scraped off the culture dishes, washed once in PBS, and ultrasonically disrupted in redistilled water. Duplicate samples of the homogenate were analyzed fluorometrically for DNA (20, 21).

Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Trypsinized cells were incubated for 45 min with 5 μM quin 2, which resulted in a loading of $\sim$ 2–4 nmol quin 2/10^6 cells. Cell suspensions were washed twice before being resuspended in 1.3 ml medium, consisting of a Hepes buffer (pH 7.4) with 1.28 mM Ca^{2+}, Cl^- as the sole anion and 1 mg/ml albumin (22). The excitation and emission wavelengths were 340 and 490 nm, respectively, and calibration was performed as previously described (23). Experiments were carried out at 37°C using polystyrene cuvettes in a spectrofluorometer (Perkin-Elmer Corp. Norwalk, CT), allowing constant stirring. All traces shown are typical for experiments repeated with at least three different cell preparations.

Membrane potential. Qualitative changes in membrane potential were measured with the fluorescent dye bisoxonol (24). The excitation and emission wavelengths were set at 540 and 580 nm, respectively, and bisoxonol (final concentration 150 nM) was allowed to equilibrate with 1.0 ml of buffer solution before adding $\sim$ 0.5 $\times$ 10^6 cells.

Ca^{2+} channels. The whole-cell configuration of the patch-clamp technique was used to measure Ca^{2+}-currents under voltage-clamp conditions (25). During the experiments the pipette solution consisted of (in mM): 150 *N*-methyl-D-glucamine, 10 EGTA, 2 $CaCl_2$, 1 $MgCl_2$, 5 Hepes (pH 7.15), 3 Mg-ATP, and 130 HCl. The bath solution during these experiments was composed of (in mM): 138 NaCl, 10.2 $CaCl_2$, 5 Hepes (pH 7.4), 5.6 KCl, 1.2 $MgCl_2$ and 1 μg/ml tetrodotoxin to block Na^+ currents. The experiments were performed at room temperature (20–23°C). A patch-clamp amplifier (model EPC-7 List Electronic, Darmstadt, Germany) was used to record the current. Pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany). Voltage pulses and ramps were produced by a Compaq Portable III computer using the program pClamp (Axon Instruments, Burlingame, CA). Current signals were filtered at 3 kHz (−3 dB), amplified, and digitized at 2 kHz. Leakage and capacitive transients were removed by subtracting the scaled response to a 20-mV pulse.

For measurements of Ca^{2+} channel mRNA, RNA was prepared from $\sim$ 10^8 RINm5F cells by guanidinium isothiocyanate/CsCl centrifugation (26). PolyA^+ RNA was selected using the fast-track kit. For Northern analysis, 4 μg polyA^+ RNA was denatured with 2.2 M formaldehyde and electrophoresed on 0.8% agarose gels (27). The Ca^{2+} channel probe (28) and a γ-actin probe (reference 29; serving as control) were P-labeled using Megaprime and used for hybridization at 42°C in the presence of 50% formamide. Blots were autoradiographed and the intensities of the appropriate bands were assessed by densitometry.

Inositol phosphate production. Cell cultures were maintained in RPMI 1640 for 4 d. During the last 24 h of culture, 16 μCi/ml [2-^{3}H]-inositol was present in media. After washing twice, 1–2 $\times$ 10^6 cells were incubated at 37°C for 60 s in a buffer (18) containing 1.28 mM Ca^{2+} and 11.1 mM glucose in the presence or absence of 100 μM carbamylcholine. After termination of incubations with trichloroacetic acid, inositol phosphate production was assayed using ion exchange chromatography as described (30, 31).

Endogenous protein kinase C activity. Cells were labeled with 1 mCi/ml ^{32}P for 3 h in a bicarbonate buffer (18) containing 10 mM Hepes, 17 mM glucose, 0.1 mM phosphate, 1 mg/ml albumin and 1% fetal calf serum. During the final 10 min of labeling, TPA or carbamylcholine were present or absent in the buffer. The cells were then washed in ice-cold bicarbonate buffer, scraped off the dishes, and lyzed in 100 μl 150 mM NaCl, 50 mM Tris (pH 7.4), 0.5% Nonidet-40, 0.1% SDS, 5 mM EDTA, 100 kU/ml Trasylol, and 10 mM benzamidine. After

sedimentation of nuclei by centrifugation, monoclonal antibodies directed against an 80–87 kD endogenous substrate (32) were added. This substrate was originally isolated from rat brain and has been shown to be specifically phosphorylated by protein kinase C (32). After 30 min, immunocomplexes were precipitated using protein A–Sepharose. The immunoprecipitated products were extensively washed before being subjected to either 1-dimensional (33) or 2-dimensional gel electrophoresis (34) and subsequent autoradiography.

Binding of scopolamine and glibenclamide. For studies of binding of scopolamine to muscarinic receptors, intact cell cultures were washed in PBS and incubated for 5 min at 37°C in a bicarbonate buffer (18) containing 11.1 mM glucose, 10 mM Hepes, and 85 nCi/ml (1 nM) [^{3}H]*N*-methyl-scopolamine. Cells were then scraped off culture dishes, placed onto Whatman glassfibre filters and remaining free isotope eliminated by extensive washing of filters with bicarbonate buffer. Sulfonylurea binding was analyzed by incubating cells that had been scraped off culture dishes with [^{3}H]glibenclamide (0.3 μCi/ml) for 2 h at 0–4°C. The cells were then placed onto Whatman glassfiber filters and the remaining free isotope was eliminated by extensive washing of filters with bicarbonate buffer. Specific binding of [^{3}H]*N*-methyl-scopolamine and [^{3}H]glibenclamide was calculated as the difference between total binding and that in the presence of a 1,000-fold excess of unlabeled ligand. Radioactivity was quantified by scintillation counting after addition of 10 ml Instagel.

Statistical analysis. Means±SEM were calculated and groups of data were compared using Student's paired or unpaired *t* test. In case of multiple comparisons, data were evaluated by two-way factorial ANOVA in conjunction with Bonferroni's modified *t* statistics by use of a StatView™ 512+ (Version 1.0) software package from Abacus Concepts (Berkeley, CA) and BrainPower, Inc. (Calabasas, CA).

Results

Contents of DNA, insulin, polyamines, and ATP. Proliferation of RINm5F cells was markedly retarded by 4 d of DFMO exposure, as evidenced by a decreased DNA content per dish (Table I). Cell viability, as measured by Trypan blue exclusion, was however only slightly reduced (96.3±2.1% Trypan blue negative control cells vs 88.7±4.3% in the DFMO group). The cellular content of polyamines was profoundly decreased by DFMO; the decreases in putrescine and spermidine being the most prominent (Table I). In parallel, the cellular contents of insulin and ATP were significantly increased by the inhibitor (Table I).

Insulin secretion. Insulin secretion from RINm5F cells in response to various secretagogues in static incubations is shown in Table II. Under all incubation conditions, DFMO-treated cells exhibited a two- to threefold greater insulin release as compared to untreated cells. Untreated cells were not sensitive to stimulation with high glucose, K^+, glibenclamide, or carbamylcholine but displayed a slightly increased insulin release in response to theophylline and TPA. DFMO-treated cells were, however, sensitized to some secretagogues. In particular K^+ and TPA were effective in this respect, evoking a three- to fourfold and two- to threefold increase, respectively, of insulin release as compared to DFMO-treated cells incubated with 2.8 mM glucose only. Carbamylcholine also, albeit to a lesser extent, elicited an increased insulin output from DFMO-treated cells, whereas theophylline enhanced the insulin release to about the same degree as in untreated cells. Glibenclamide and high glucose concentrations (28 mM) failed to increase insulin secretion from DFMO-exposed cells. Neither was the increase in insulin release between 0 and 2.8 mM glucose greater in DFMO-treated cells than in control cells during 30 min of incubation (not shown). This latter experiment was performed after a 45-min preincubation period at 0 mM glucose.

Table I. Effects of DFMO on Contents of DNA, Polyamines, Insulin, and ATP in RINm5F Cultures

	Cell culture	
	Control	DFMO
DNA (μg/well)	57±7 (5)	21±3 (5)*
Putrescine (pmol/10^6 cells)	96±14 (4)	4±1 (4)‡
Spermidine (pmol/10^6 cells)	2,945±190 (4)	88±10 (4)‡
Spermine (pmol/10^6 cells)	1,846±178 (4)	610±84 (4)‡
Insulin (ng/μg DNA)	6±0.7 (6)	27±4 (6)*
ATP (pmol/μg DNA)	43±2.3 (4)	95.2±7.4 (4)*

Cells were cultured for 4 d with or without the addition of 1 mM DFMO. Values are means±SEM for the number of observations in parentheses. DNA was measured fluorometrically. Polyamines were extracted in $HClO_4$ and measured fluorometrically after TLC separation of dansylated compounds. The amount of insulin in acid ethanol extracts was measured radioimmunologically. ATP was determined by use of a bioluminescence kit. * and ‡ denote $P < 0.01$ and $P < 0.001$ vs control cells using Student's paired *t* test.

$[Ca^{2+}]_i$ and membrane potential. Since the process of insulin secretion is controlled largely by alterations in $[Ca^{2+}]_i$ (35), we decided to investigate whether the increased insulin release in DFMO-treated cells was paralleled by an altered handling of this divalent cation. Fig. 1 shows fluorescence recordings from experiments where effects of K^+, TPA, the sulfonylurea tolbutamide, and the sulfonamide diazoxide were studied in RINm5F cells cultured with or without 1 mM DFMO for 4 d.

Table II. Enhanced Effect of Secretagogues on Insulin Release from DFMO-treated Insulinoma Cells

	Insulin release	
Secretagogue	Untreated	DFMO-treated
	pg/μg DNA per 30 min	
First series		
Control (2.8 mM glucose)	460±61 (10)	1,230±110 (10)§
Theophylline (10 mM)	660±84 (10)*	1,610±160 (10)‡§
KCl (25 mM)	530±54 (10)	4,060±840 (10)‡§
Second series		
Control (2.8 mM glucose)	350±30 (8)	770±84 (8)§
Glucose (28 mM)	380±31 (8)	780±69 (8)§
TPA (10 nM)	460±38 (8)*	2,070±280 (8)‡§
Glibenclamide (5 μg/ml)	350±31 (8)	840±80 (8)§
Carbamylcholine (100 μM)	370±15 (8)	1,000±110 (8)‡§

Cells were cultured for 4 d with or without the addition of 1 mM DFMO. After preincubation for 45 min in a bicarbonate buffer with 2.8 mM glucose, cells were incubated for another 30 min in fresh buffer containing 2.8 mM glucose plus the desired secretagogue. The amount of insulin released was measured radioimmunologically. Values are means±SEM for the number of observations in parentheses. Statistical analysis of differences between groups was performed by two-way factorial ANOVA in conjunction with Bonferroni's modified *t* statistics. * and ‡ denote $P < 0.01$ and $P < 0.001$ vs cells of the same culture condition incubated in 2.8 mM glucose only; § denotes $P < 0.001$ vs untreated cells incubated with the same secretagogue.

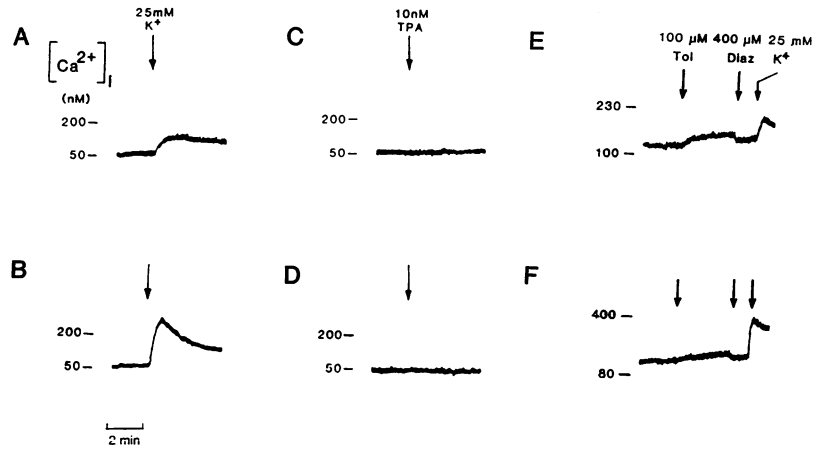

Figure 1. Effects of K^+ (*A* and *B*), TPA (*C* and *D*), tolbutamide (*Tol*), and diazoxide (*Diaz*) (*E* and *F*) on $[Ca^{2+}]_i$ as monitored by the fluorescent indicator quin 2 in control (upper traces) and DFMO-treated cells (lower traces). A basal concentration of 2.8 mM glucose was present throughout all experiments.

Addition of 25 mM K^+ to control cells (Fig. 1 *A*) caused a prompt elevation in $[Ca^{2+}]_i$, the mean maximal increase reaching 98±26% over basal level (n = 4). In DFMO-treated cells (Fig. 1 *B*), this increase was much more pronounced, corresponding to a mean stimulation of 432±52% (n = 4; $P < 0.001$ vs control cells; Student's unpaired t test). In both DFMO-treated and control cells the K^+-induced increase in $[Ca^{2+}]_i$ was completely reversed by the addition of 50 μM D-600, a blocker of voltage-activated Ca^{2+} channels (not shown). The phorbol ester TPA, at 10 nM, failed to influence basal $[Ca^{2+}]_i$ both in control (Fig. 1 *C*) and in DFMO-treated cells (Fig. 1 *D*). Tolbutamide (100 μM) slightly increased $[Ca^{2+}]_i$ to approximately the same degree in control (Fig. 1 *E*) and DFMO-treated (Fig. 1 *F*) cells. In the same figure, it is demonstrated that the hyperglycemic sulfonamide diazoxide, known to open ATP-regulated K^+-channels (36), counteracted the increases in $[Ca^{2+}]_i$ elicited by the sulfonylurea in both controls and DFMO-treated cells (Fig. 1, *E* and *F*). The $[Ca^{2+}]_i$ response to the Ca^{2+} ionophore ionomycin (2 μM) was not different between control and polyamine-deficient cells, arguing against a decreased buffering of Ca^{2+} in the DFMO-treated cells (not shown). In Fig. 2 *A*, it is shown that 100 μM carbamylcholine evoked an increase in $[Ca^{2+}]_i$ in control cells (87±19%, n = 4), an effect that was more pronounced in DFMO-treated cells (Fig. 2 *B*) in which the increase was 490±65% (n = 4; $P < 0.001$ vs control cells). To test the possibility that the effects of carbamylcholine on $[Ca^{2+}]_i$ were partially caused by depolarization, cells were pretreated for 2 min with the Ca^{2+}-channel blocker D-600 (50 μM) before and during carbamylcholine exposure. This procedure resulted in a complete inhibition of the increase in $[Ca^{2+}]_i$ in control cells (Fig. 2 *C*), whereas no such attenuation was observed in DFMO-treated cells (Fig. 2 *D*). Fig. 2 *E* shows alterations in membrane potential evoked by carbamylcholine and K^+ in control cells. The depolarizing effect of carbamylcholine, but not of K^+, seemed more pronounced in DFMO-treated cells (Fig. 2 *F*).

Ca^{2+} channels. The effects of DFMO on Ca^{2+} influx across the plasma membrane via voltage-activated Ca^{2+}-channels was explored by patch-clamp analysis using the whole-cell configuration. Biphasic current(I)-voltage(V) curves were obtained in a few cells in both the control group and the DFMO-treated group. This suggests that the RINm5F cell, in analogy to what seems to be the case in the normal rat pancreatic β cell (37), is equipped with two types of Ca^{2+}-channels; L and T type. However, there is no evidence that DFMO treatment preferentially increased the conductivity or number of either of these channels. The currents elicited by depolarizing voltage commands going from a holding potential of −80 mV to potentials between −60 and 0 mV in control and DFMO-treated RINm5F cells are shown in Fig. 3, *A* and *B*. Membrane depolarization resulted in the development of inward currents, which were larger in the DFMO-treated cells. In addition, there was a tendency towards a more pronounced inactivation of the channels in these latter cells. The results are summarized in Fig. 3 *C*. The I-V relationships make it evident that the currents elicited in

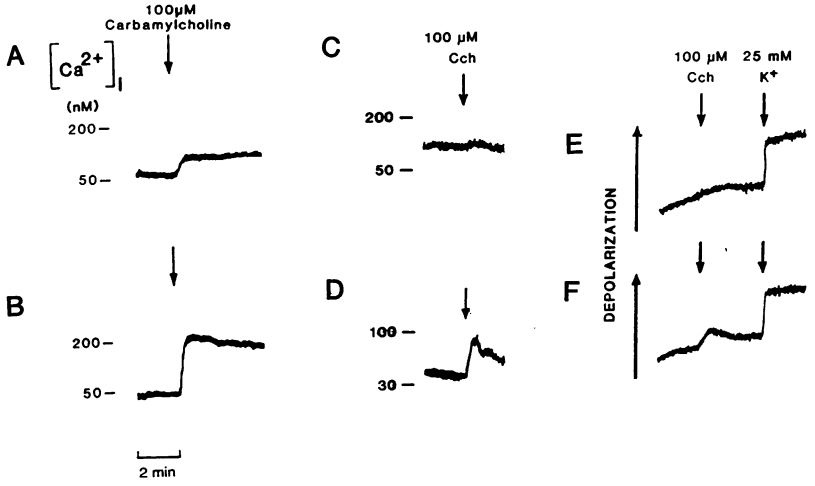

Figure 2. Effects of carbamylcholine (*Cch*) and K^+ on $[Ca^{2+}]_i$ and membrane potential, as measured by fluorescence changes in cells loaded with quin 2 and bisoxonol, respectively. Cells in groups *C–F* were treated with the calcium channel blocker D-600 (50 μM) for 2 min before the onset of the experiment and throughout the remainder of the experiment. A basal concentration of 2.8 mM glucose was present during all experiments. $[Ca^{2+}]_i$ in untreated (*A* and *C*) and DFMO-treated (*B* and *D*) cells. Qualitative changes in membrane potential are shown in untreated (*E*) and DFMO-treated (*F*) cells.

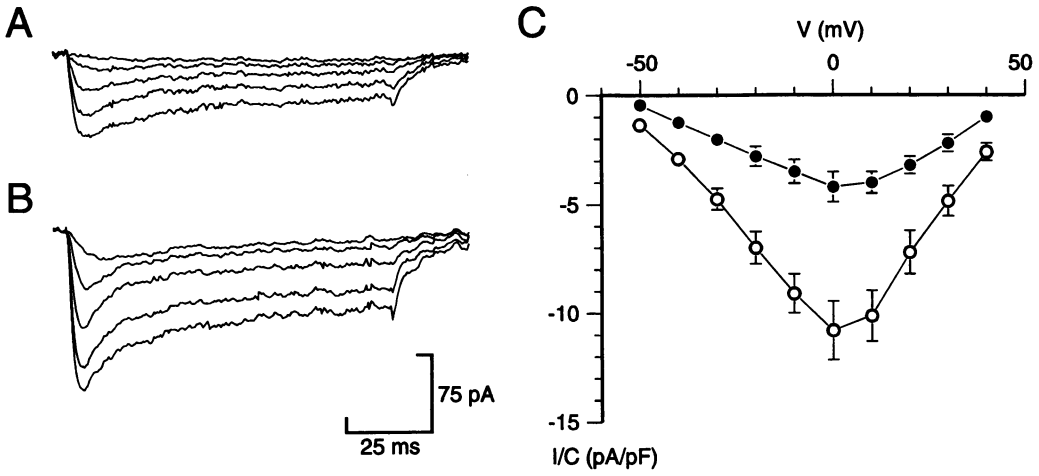

Figure 3. Increased whole cell Ca^{2+} currents in DFMO-treated cells. (*A*) Whole cell Ca^{2+} currents in control cells elicited by voltage steps from a holding potential of −80 mV to indicated potentials are shown. (*B*) Ca^{2+} currents elicited by depolarizing voltages as in *A* in DFMO-treated cells. Pulse protocol for A and B is given at the top. (*C*) Whole cell current(I)-voltage(V) relations for control cells (○) and DFMO-treated cells (●). Currents are normalized to the cell surface area (as estimated from measurements of cell capacitance) and therefore given as pA/pF. Differences are $P < 0.001$ (Student's unpaired *t* test) for all points except those of the two most positive potentials where it is $P < 0.01$. Both curves represent mean values±SEM. for 14 cells.

the DFMO-treated cells are substantially stronger than those seen in control cells. Currents were expressed in relation to the cell surface area, which was estimated from measurements of cell capacitance. The capacitance for control cells was 16.0±0.7 pF, and for DFMO-treated cells it was 10.8±1.5 pF (n = 14; $P < 0.01$ vs controls; Student's unpaired *t* test), indicating a considerable diminuation in cell size after DFMO treatment.

Northern analysis revealed that DFMO treatment did not change RINm5F cell content of calcium channel mRNA. Thus, in two experiments (results expressed as arbitrary units normalized against γ-actin mRNA) control cells contained 4.33 and 6.60 U of calcium channel mRNA, whereas DFMO-treated cells contained 4.53 and 6.67 U. The calcium channel mRNA was identified as an 11-kb band on the blots, while γ-actin mRNA was 2 kb in size.

Inositol phosphate production. Since DFMO treatment resulted in an increased $[Ca^{2+}]_i$ response to carbamylcholine, it became worthwhile to determine whether this effect could be explained by an increased phosphatidylinositol-specific phospholipase C activity. Thus, the effect of carbamylcholine on the production of inositol phosphates was assessed. As shown in Table III, the basal synthesis of the different inositol phosphates did not differ between control and DFMO-treated cells. Furthermore, stimulation of untreated RINm5F cells with 100 μM carbamylcholine for 1 min failed to significantly influence the rate of synthesis of inositol phosphates. In contrast, the synthesis of $InsP_2$ and $InsP_3$ was markedly enhanced by carbamylcholine in DFMO-treated cells.

Endogenous protein kinase C activity. Given the generally recognized role of TPA as a protein kinase C stimulator (38) and the improved secretory response to the phorbol ester in DFMO-treated cells, it became relevant to determine if the endogenous C-kinase activity was changed by DFMO exposure. Fig. 4 shows that the activity of protein kinase C, as measured by ^{32}P-labeling of a C-kinase–specific substrate (32), under basal conditions did not differ between control and DFMO-treated cells. There was neither any difference detected between the two groups in the degree of stimulation by TPA, which in both controls and DFMO-treated cells caused a marked and equal increase in C-kinase activity. Somewhat surprisingly, carbamylcholine (10–100 μM) did not affect protein kinase C activity in the RINm5F cells, either in control or in DFMO-treated cells (Fig. 5), although TPA was effective also in this series of experiments (not shown).

Binding of glibenclamide and scopolamine. As shown in Table IV, specific binding of the hypoglycemic sulfonylurea glibenclamide to RINm5F cells was increased by ~ 60% in DFMO-treated cells, suggesting a lack of correlation between binding of the drug and subsequent cellular responses. In contrast, the binding affinity of the muscarinic receptor ligand *N*-methyl-scopolamine to RINm5F cell membranes was found not to be changed by DFMO exposure (Table IV).

Discussion

The present investigation was conducted with two overall aims in mind. Since DFMO treatment causes cessation of insulinoma cell proliferation, the drug could be used as a tool to assess the effects of decreased RINm5F cell proliferation on the stimulus-secretion coupling of insulin release by the tumoral cells. Alternatively, since DFMO specifically causes partial depletion of polyamine contents, the role of polyamines for certain steps in the stimulus-secretion coupling could also be investigated. The results show that RINm5F cells partially depleted in polyamines by DFMO increase their contents of insulin and ATP. These cells also exhibit an enhanced responsiveness of the stimulus-secretion coupling to certain secretagogues. The DFMO-treated cells were particularly sensitized to K^+-induced depolarization, which resulted in marked increases in both $[Ca^{2+}]_i$ and insulin secretion. Voltage-clamp experiments revealed that these effects were paralleled by increased voltage-activated Ca^{2+} currents, findings in line with a previous report describing inhibitory actions of polyamines on Ca^{2+} channel activity in rat brain (39). Because the cellular content of Ca^{2+} channel mRNA was not altered by DFMO, it is likely that the augmented current reflects an increased activity of individual channels rather than an increased number of channels.

In untreated cells, there was no detectable stimulation of insulin secretion in response to the muscarinic agonist carbamylcholine, although a modest increase in $[Ca^{2+}]_i$ was noted. This increase seemed to originate from a depolarizing effect of carbamylcholine with a subsequent Ca^{2+} influx, because the increase in $[Ca^{2+}]_i$ was completely attenuated by the blocker of

Table III. Increased Production of Inositol Phosphates in DFMO-treated Insulinoma Cells

Culture	Incubation	Inositol phosphate production (dpm/μg DNA)		
		$InsP_1$	$InsP_2$	$InsP_3$
		dpm/μg DNA		
Control	Control	1,985±692	283±62	264±63
Control	Carbamylcholine	2,135±851	233±57	313±69
DFMO	Control	2,186±612	268±58	284±40
DFMO	Carbamylcholine	3,825±1493	510±138*	539±129*

Cells were cultured for 4 d with or without the addition of 1 mM DFMO. During the final 24 h of culture, 16 μCi/ml of [^{3}H]inositol was present in media. At the end of the culture period, cells were incubated for 60 s in the presence or absence of 100 μM carbamylcholine. The production of different inositol phosphates was subsequently analyzed by ion exchange chromatography. Values are means±SEM for six experiments. * denotes $P < 0.05$ vs. DFMO-treated cells incubated without carbamylcholine using Student's paired t test. $InsP_1$, inositol monophosphate; $InsP_2$, inositol biphosphate.

voltage-activated Ca^{2+} channels D-600. The $[Ca^{2+}]_i$ increase was much more pronounced after carbamylcholine exposure in DFMO-treated cells and, in contrast to untreated cells, could not be abolished by D-600. The possibility of an altered specificity of D-600 in DFMO-treated cells was excluded by the finding that the K^+-mediated increase in $[Ca^{2+}]_i$ was totally attenuated by D-600. The enhanced $[Ca^{2+}]_i$ increase in response to carbamylcholine in DFMO-treated cells was paralleled by a significantly increased production of inositol trisphosphate ($InsP_3$), which presumably caused most of the observed increase in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from the endoplasmic reticulum (40). The changes in $[Ca^{2+}]_i$ and $InsP_3$ production taken together suggest that the responsiveness of phospholipase C becomes enhanced in polyamine-deficient cells, although a decreased $InsP_3$ degradation cannot be completely excluded. This increased sensitivity of the enzyme apparently occurs at the postreceptor level, since binding of the muscarinic receptor ligand *N*-methyl-scopolamine was not altered by DFMO. Since phospholipase C catalyzes the formation of not only $InsP_3$, but also the endogenous protein kinase C activator diacylglycerol (41), it became relevant to determine whether some of the effects of carbamylcholine on insulin secretion might have been mediated via the protein kinase C system. Our findings, however, disclosed that there was no activation of this enzyme by carbamylcholine either in untreated or DFMO-treated cells. These results reinforce the findings of a recent report (42), showing that not in all instances is a stimulated $InsP_3$ synthesis coinciding with protein kinase C activation. The observations also point to an increased $InsP_3$ generation as the locus of enhancement of carbamylcholine responsiveness, rather than the protein kinase C pathway. Nonetheless, our finding of an enhanced insulin release in DFMO-treated cells in response to the tumor promoter TPA suggests that polyamine depletion confers an enhanced responsiveness of the protein kinase C system to phorbol esters. The action of TPA at the low concentration used here is considered specifically attributable to C-kinase activation (38). The protein kinase C system is believed to be involved in the regulation of insulin secretion by making the secretory system more sensitive to Ca^{2+} (43), and has been shown to phosphorylate insulin granule membrane proteins (44). The C-kinase system in polyamine-deficient cells seemed, however, to have been sensitized at a step beyond the actual phosphorylation event since the labeling of an immunoprecipitable C-kinase specific substrate did not differ between control and DFMO-cultured cells after short term TPA exposure. It is thus possible that some effector or target proteins distal in the protein kinase C signaling cascade become sensitized to C-kinase activation in DFMO-treated cells. In the present study, we could not reproduce the previously reported lowering effect of TPA on $[Ca^{2+}]_i$ (43), suggesting that this effect is not consistently present in all subclones of the RINm5F cell line.

In recent reports polyamines were assigned an inhibitory role in phospholipase C-catalyzed polyphosphoinositide hydrolysis (45, 47). This feature may reflect that polyamines, by virtue of their polycationic nature, compete with Ca^{2+} for binding sites on phosphatidylinositol 4,5-bisphosphate, which is the major phospholipase C substrate. Thus, in cells partially depleted in polyamines, phospholipase C becomes relieved from the normal inhibitory action of polyamines and an increased sensitivity to secretory stimulation is acquired. However, it cannot be ruled out that the improved sensitivity of the secretory apparatus to some stimuli merely is caused by the increased insulin and ATP contents of these cells, rather than polyamine deficiency per se.

The lack of a normal insulin secretory response to glucose

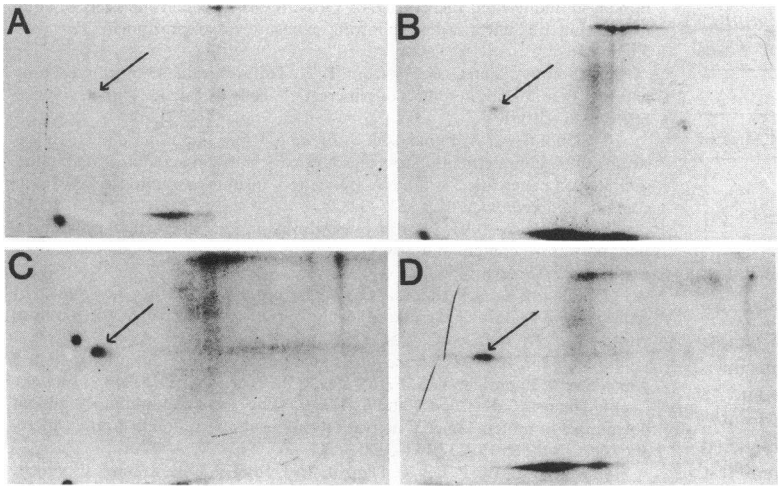

Figure 4. Endogenous C-kinase activity. Cells cultured in the absence (*A* and *C*) or presence (*B* and *D*) of DFMO were labeled with ^{32}P and incubated for 10 min with (*C* and *D*) or without (*A* and *B*) TPA. Cell homogenates were incubated with antibodies directed against an 80–87-kD specific protein kinase C substrate (*arrows*). Immunoprecipitated products were subjected to 2-d gel electrophoresis before autoradiography. The autoradiograph shown is representative of three separate experiments.

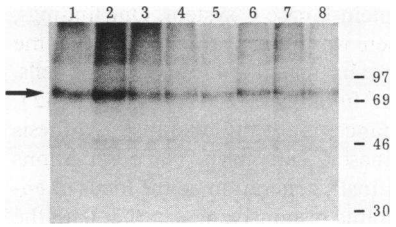

Figure 5. Carbamylcholine fails to affect C-kinase activity in RINm5F cells. Cells cultured in the absence (lanes *1–4*) or presence (lanes *5–8*) of DFMO were labeled with ^{32}P and incubated for 10 min with different concentrations of carbamylcholine (lanes *1* and *5*, control; lanes *2* and *6*, 10 μM; lanes *3* and *7*, 50 μM; lanes *4* and *8*, 100 μM). Cell homogenates were incubated with antibodies directed against an 80-87 kDa specific protein kinase C substrate (*arrow*). Immunoprecipitated products were subjected to 1-d gel electrophoresis before autoradiography.

in the RINm5F cells observed in this study confirms findings by other investigators (48). In contrast to the impact of DFMO-treatment on Ca^{2+} currents, there was a lack of increased responsiveness to glucose and hypoglycemic sulfonylureas. These substances are believed to exert their secretagogic action via closure of ATP-sensitive K^+ channels followed by Ca^{2+} influx (49, 50). Since our results indicate that neither the $[Ca^{2+}]_i$ increase nor insulin secretion in response to high glucose or the sulfonylureas tolbutamide or glibenclamide were enhanced in DFMO-treated cells, this is taken to indicate that ATP-regulated K^+ channel activity remained unchanged after the decrease in polyamine content, in spite of increased sulfonylurea binding. The RINm5F cells exhibit a low rate of glucose oxidation and a low content of ATP compared to native β cells, and it is conceivable that the very small amount of ATP generated by glucose oxidation, even after DFMO exposure (16), is inadequate to induce insulin release in a normal dose-response interval in these cells. It was presently also noted that DFMO-treated cells increased their output of insulin in response to the phosphodiesterase inhibitor theophylline to the same extent as untreated cells. This finding suggests a lack of sensitization of the stimulus-secretion coupling to the cyclic AMP system, in contrast to the Ca^{2+} and C-kinase dependent secretory responses. Although DFMO is a totally specific inhibitor of ornithine decarboxylase (15), it cannot be excluded that some of the effects reported here might result from possible indirect changes in pathways of ornithine metabolism other than polyamine synthesis, such as the urea cycle.

Table IV. Effects of DFMO on Binding of N-methyl-scopolamine and Glibenclamide in RINm5F Cultures

	Cell culture	
	Untreated	DFMO-treated
N-methyl-scopolamine binding (dpm/10^5 cells)	50±4.1 (4)	43±4.8 (4)
Glibenclamide binding (dpm/μg DNA)	374±57 (5)	612±70 (5)*

Cells were cultured for 4 d with or without the addition of 1 mM DFMO. Values are means±SEM for the number of observations in parentheses. Specific binding of [^{3}H]*N*-methyl-scopolamine and [^{3}H]glibenclamide was calculated as the difference between total binding and that in the presence of a 1,000-fold excess of unlabeled ligand. * denotes $P < 0.05$ vs control cells using Student's paired *t* test.

In their entirety, our present results indicate that the behavior of the stimulus-secretion coupling of polyamine-depleted RINm5F insulinoma cells changes towards that of native β cells, thus improving the usefulness of this cell line for studies of β cell insulin secretion.

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References

1. Pegg, A. E. 1988. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* 48:759–774.

2. Danzin, C., and P. S. Mamont. 1987. Polyamine inhibition in vivo and in organ growth and repair. *In* Inhibition of Polyamine Metabolism. P. P. McCann, A. E. Pegg, and A. Sjoerdsma, editors. Academic Press, Orlando, FL. pp. 141–164.

3. Hougaard, D. M., J. H. Nielsen, and L.-I. Larsson. 1986. Localization and biosynthesis of polyamines in insulin-producing cells. *Biochem. J.* 238:43–47.

4. Hougaard, D. M., and L.-I. Larsson. 1986. Localization and possible function of polyamines in protein and peptide secreting cells. *Med. Biol.* 64:89–94.

5. Welsh, N., and Å. Sjöholm. 1988. Polyamines and insulin production in isolated mouse pancreatic islets. *Biochem. J.* 252:701–707.

6. Sjöholm, Å., N. Welsh, S. Sandler, and C. Hellerström. 1990. Role of polyamines in mitogenic and secretory responses of pancreatic β-cells to growth factors. *Am. J. Physiol.* 259:C828–C833.

7. Welsh, N. 1990. A role for polyamines in glucose-stimulated insulin-gene expression. *Biochem. J.* 271:393–397.

8. Dunger, A.-M., Å. Sjöholm, and D. L. Eizirik. 1990. Amino acids and human amniotic fluid increase DNA biosynthesis in pancreatic islets of adult mouse, but this effect is lost following exposure to streptozotocin. *Pancreas.* 5:639–646.

9. Sjöholm, Å., and C. Hellerström. 1991. TGF-β stimulates insulin secretion and blocks mitogenic response of pancreatic β-cells to glucose. *Am. J. Physiol.* 260:C1046–C1051.

10. Sjöholm, Å., A. Funakoshi, S. Efendić, C.-G. Östenson, and C. Hellerström. 1991. Long-term inhibitory effects of pancreastatin and diazepam binding inhibitor on pancreatic β-cell DNA replication, insulin secretion and polyamine content. *Endocrinology.* 128:3277–3282.

11. Sjöholm, Å. 1991. Phorbol ester stimulation of pancreatic β-cell replication, polyamine content and insulin secretion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 294:257–260.

12. Sjöholm, Å., N. Welsh, and C. Hellerström. 1992. Lithium increases DNA replication, polyamine content and insulin secretion by rat pancreatic β-cells in vitro. *Am. J. Physiol.* 262:C391–C395.

13. Sjöholm, Å. 1992. Intracellular signal transduction pathways that control pancreatic β-cell proliferation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 311:85–90.

14. Thams, P., K. Capito, and C. J. Hedeskov. 1986. An inhibitory role for polyamines in protein kinase C activation and insulin secretion in mouse pancreatic islets. *Biochem. J.* 237:131–138.

15. Metcalf, B. W., P. Bey, C. Danzin, M. J. Jung, P. Casara, and J. P. Vevert.

1978. Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues. *J. Am. Chem. Soc.* 100:2551–2553.

16. Sjöholm, Å., N. Welsh, V. Hoftiezer, P. W. Bankston, and C. Hellerström. 1991. Increased glucose oxidation and contents of insulin and ATP in polyamine-depleted rat insulinoma cells (RINm5F). *Biochem. J.* 277:533–540.

17. Seiler, N. 1983. Liquid chromatographic methods for assaying polyamines using prechromatographic derivatization. *Methods Enzymol.* 94:10–20.

18. Krebs, H. A., and K. Henseleit. 1932. Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z. Physiol. Chem.* 210:33–66.

19. Heding, L. G. 1972. Determination of total serum insulin (IRI) in insulin-treated diabetic patients. *Diabetologia.* 8:260–266.

20. Kissane, J. M., and E. Robins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the nervous system. *J. Biol. Chem.* 233:184–188.

21. Hinegardner, R. T. 1971. An improved fluorometric assay for DNA. *Anal. Biochem.* 39:197–201.

22. Hellman, B. 1975. The significance of calcium for glucose stimulation of insulin release. *Endocrinology.* 97:392–398.

23. Hesketh, T. R., G. A. Smith, J. P. Moore, M. V. Taylor, and J. C. Metcalfe. 1983. Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. *J. Biol. Chem.* 258:4876–4882.

24. Rink, T. J., C. Montecucco, T. R. Hesketh, and R. Y. Tsien. 1980. Lymphocyte membrane potential assessed with fluorescent probes. *Biochim. Biophys. Acta.* 595:15–30.

25. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* 391:85–100.

26. Chirgwin, J. M., A. E. Przybyla, R. J. McDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *In* Molecular Cloning: A Laboratory Manual. N. Nolan, Editor. Cold Spring Harbor Laboratory Press, 7.43–7.45.

28. Seino, S., L. Chen, M. Seino, O. Blondel, J. Takeda, J. H. Johnson, and G. I. Bell. 1992. Cloning of the alpha 1 subunit of a voltage-dependent calcium channel expressed in pancreatic beta cells. *Proc. Natl. Acad. Sci. USA.* 89:584–588.

29. Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* 3:787–795.

30. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* 212:473–482.

31. Nilsson, T., P. Arkhammar, A. Hallberg, B. Hellman, and P.-O. Berggren. 1987. Characterization of the inositol 1,4,5-trisphosphate-induced Ca^{2+} release in pancreatic β-cells. *Biochem. J.* 248:329–336.

32. Albert, K., S. I. Walaas, J. K.-T. Wang, and P. Greengard. 1986. Widespread occurrence of "87 kDa" protein, a major specific substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA.* 83:2822–2826.

33. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

34. Perdew, G. H., H. W. Schaup, and D. P. Selivonchick. 1983. The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. *Anal. Biochem.* 135:453–455.

35. Wollheim, C. B., and G. W. G. Sharp. 1981. Regulation of insulin release by calcium. *Physiol. Rev.* 61:914–973.

36. Trube, G., P. Rorsman, and T. Ohno-Shosaku. 1986. Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pfluegers Arch. Eur. J. Physiol.* 407:493–499.

37. Hiriart, I., and P. Mattesen. 1988. Na channels and two types of Ca channels in rat pancreatic B-cells identified with the reverse hemolytic plaque assay. *J. Gen. Physiol.* 91:617–639.

38. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693–698.

39. Schoemaker, H. 1992. Polyamines allosterically modulate [^{3}H]-nitrendipine binding to the voltage-sensitive calcium channel in rat brain. *Eur. J. Pharmacol. (Mol. Pharm. Sect.)* 225:167–169.

40. Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature (Lond.).* 361:315–325.

41. Kikkawa, U., A. Kishimoto, and Y. Nishizuka. 1989. The protein kinase C family: heterogeneity and its implications. *Ann. Rev. Biochem.* 58:31–44.

42. Gonzales, F. A., E. Rozengurt, and L. A. Heppel. 1989. Extracellular ATP induces the release of calcium from intracellular stores without the activation of protein kinase C in Swiss 3T6 mouse fibroblasts. *Proc. Natl. Acad. Sci. USA.* 86:4530–4534.

43. Arkhammar, P., T. Nilsson, and P.-O. Berggren. 1986. Stimulation of insulin release by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate in the clonal cell line RINm5F despite a lowering of the free cytoplasmatic Ca^{2+} concentration. *Biochim. Biophys. Acta.* 887:236–241.

44. Hutton, J. C., M. Peshavaria, and K. W. Brocklehurst. 1984. Phorbol ester stimulation of insulin release and secretory-granule protein phosphorylation in a transplantable rat insulinoma. *Biochem. J.* 224:483–490.

45. Kimura, T., K. Imamura, L. Eckhardt, and I. Schulz. 1986. Ca^{2+}-, phorbol ester-, and cAMP-stimulated enzyme secretion from permeabilized rat pancreatic acini. *Am. J. Physiol.* 250:G698–G708.

46. Wojcikiewicz, R. J. H., and J. N. Fain. 1988. Polyamines inhibit phospholipase C-catalyzed polyphosphoinositide hydrolysis. Studies with permeabilized GH_3 cells. *Biochem. J.* 255:1015–1021.

47. Smith, C. D., and R. Snyderman. 1988. Modulation of inositol phospholipid metabolism by polyamines. *Biochem. J.* 256:125–130.

48. Praz, G. A., P. A. Halban, C. B. Wollheim, B. Blondel, A. J. Strauss, and A. E. Renold. 1983. Regulation of immunoreactive insulin release from a rat cell line (RINm5F). *Biochem. J.* 210:345–352.

49. Ashcroft, F. M., D. E. Harrison, and S. J. H. Ashcroft. 1984. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature (Lond.).* 312:446–448.

50. Rorsman, P., P.-O. Berggren, K. Bokvist, and S. Efendić. 1990. ATP-regulated K^+ channels and diabetes mellitus. *News Physiol. Sci.* 5:143–147.