Protein kinase A translocation and insulin secretion in pancreatic β-cells: studies with adenylate cyclase toxin from *Bordetella pertussis*

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Activation of protein kinase A (cAMP-dependent protein kinase; PKA) triggers insulin secretion in the β-cell. Adenylate cyclase toxin (ACT), a bacterial exotoxin with adenylate cyclase activity, and forskolin, an activator of adenylate cyclase, both dose-dependently increased insulin secretion in the presence, but not the absence, of glucose in insulin-secreting βTC3 cells. The stimulation of cAMP release by either agent was dose-dependent but glucose-independent. Omission of extracellular Ca^{2+} totally abolished the effects of ACT on insulin secretion and cytosolic cAMP accumulation. ACT and forskolin caused rapid and dramatic increases in cytosolic Ca^{2+}, which were blocked by nifedipine and the omission of extracellular Ca^{2+}. Omission of glucose completely blocked the effects of forskolin and partially blocked the effects of ACT on cytosolic Ca^{2+}. PKA α, β and γ catalytic subunits (Cα, Cβ and Cγ respectively) were identified in βTC6 cells by confocal microscopy. Glucose and glucagon-like polypeptide-1 (GLP-1) caused translocation of Cα to the nucleus and of Cβ to the plasma membrane and the nucleus, but did not affect the distribution of Cγ. In conclusion, glucose and GLP-1 amplify insulin secretion via cAMP production and PKAβ activation.

Key words: adenylate cyclase, cAMP, glucose.

INTRODUCTION

The second messenger cAMP mediates diverse cellular processes, such as exocytosis, cell proliferation, ion transport, intermediate metabolism and gene transcription, by activation of the cAMP-dependent protein kinase (protein kinase A; PKA) [1,2]. Activation of PKA occurs when cAMP binds to the two regulatory subunits of the tetrameric PKA holoenzyme, resulting in the release of two active catalytic subunits [3–5]. Three catalytic subunit isoforms have been identified, and designated as Cα, Cβ and Cγ, each representing a specific gene product. Cα and Cβ are closely related (93% identity), whereas Cγ is 83% and 79% similar to Cα and Cβ respectively [6]. Four types of regulatory subunits have been identified, i.e. RIα, RIβ, RIIα and RIIβ. An increase in cAMP results in translocation of PKA to the nucleus, where it may regulate gene transcription [5].

The functional role of PKA in the regulation of insulin secretion by pancreatic β-cells has been established through studies with pharmacological tools [7,8]. It is currently unclear which PKA isoforms are present in β-cells. It has been reported that type I-like and type II-like PKA regulatory subunits are present in homogenates of rat islets of Langerhans, as assessed using DEAE-cellulose ion-exchange chromatography [9]. However, their molecular masses and protein sequences are unknown, and the specific type(s) of catalytic subunits in β-cells have not been studied. The endogenous substrate(s) of PKA are also unknown [10].

PKA may be involved in the activation of insulin gene transcription by glucose, although the mechanisms of this process are still unresolved [11–14]. It has been shown that enhanced insulin gene transcription requires an increase in intracellular Ca^{2+} in fetal islet β-cells [15], in the murine insulinoma cell line βTC3 [16] and in HIT-T15 cells [17], but Ca^{2+}-dependence was not demonstrated in primary rat islets [18], HIT-T15 cells [12,19] or MIN6 cells [20]. cAMP response element binding protein (CREB) is found to be present in many cell types, and mediates cAMP-induced alterations to gene transcription [21,22] by binding to the cAMP response element (CRE) of the corresponding genes. The CRE is also present in the rat insulin I gene [23]. However, in nuclear extracts of HIT cells, CREB does not bind to the CRE of the insulin gene, although it binds to the CREs of the glucagon and somatostatin genes [24]. Moreover, the stimulation of transcription by cAMP through the insulin CRE was weak in that study [24]. Thus the mechanisms of regulation of insulin transcription by glucose and glucagon-like polypeptide-1 (GLP-1), a candidate therapeutic agent for Type II diabetes [25], are still not established.

Adenylate cyclase toxin (ACT) is a single polypeptide molecule produced by *Bordetella pertussis* [26,27]. It is present predominantly in an extracytoplasmic location in the bacterium. ACT enters target cells and catalyses the production of cAMP, and thus activates PKA [26,27]. The aims of the present study were to use this pharmacological agent to investigate the mechanisms by which physiological agents stimulate insulin secretion.

EXPERIMENTAL

Insulin-secreting β-cell lines and mouse islet isolation

The insulin-secreting mouse β-cell lines βTC3 and βTC6 were maintained in culture as described previously [28]. In brief, cells

Abbreviations used: ACT, adenylate cyclase toxin; Cα (etc.), catalytic subunit α (etc.); CRE, cAMP response element; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; GLP-1, glucagon-like polypeptide-1; KRB, Krebs/Ringer bicarbonate buffer; m/c ratio, membrane/cytosol ratio; n/c ratio, nucleus/cytosol ratio; PKA, protein kinase A/cAMP-dependent protein kinase; RIα (etc.), regulatory subunit Iα (etc.).

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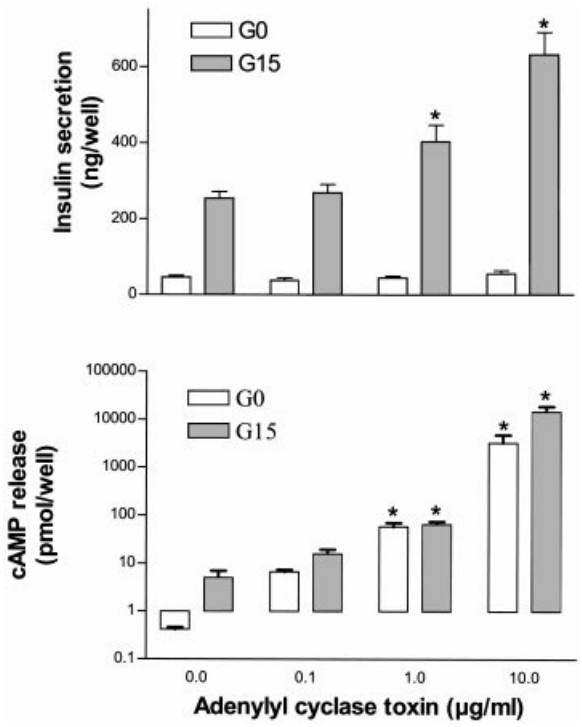

Figure 1 Effects of ACT on insulin secretion and cAMP release in βTC3 cells

βTC3 cells were plated in 24-well dishes and cultured in RPMI medium containing 11 mM glucose. On the day of the experiment, β-cells were washed twice with KRB (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM Hepes, pH 7.4, and 0.1% BSA) containing no glucose, preincubated in the absence of glucose for 60 min, and then incubated for another 60 min with ACT at the indicated concentrations in the absence (G0; open bars) or in the presence (G15; solid bars) of 15 mM glucose. Results are means $\pm$ S.E.M. for insulin secretion (upper panel) and cAMP release (lower panel) from three experiments performed in triplicate.

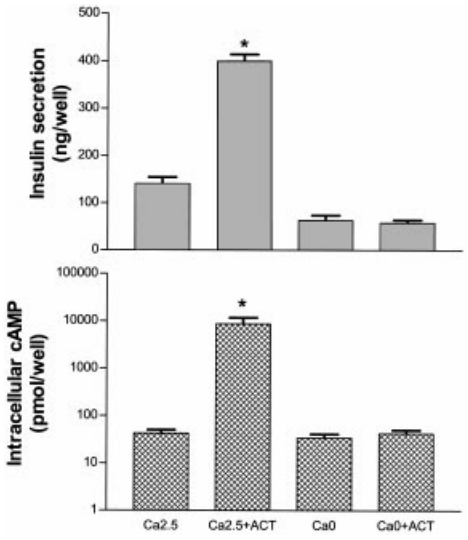

Figure 2 Ca^{2+} dependence of stimulation by ACT of insulin secretion and cAMP content in βTC3 cells

β-Cells were prepared as in Figure 1. ACT was used at 5 μg/ml in the absence (Ca0) or presence (Ca2.5) of 2.5 mM Ca^{2+} in KRB supplemented with 15 mM glucose. Incubation buffer was taken for measurement of insulin, and intracellular cAMP accumulation was determined after washing the cells and extracting cAMP with ice-cold perchloric acid. Results are means $\pm$ S.E.M. for insulin secretion (upper panel) and intracellular cAMP content (lower panel) from three experiments performed in triplicate.

were maintained in RPMI 1640 medium (βTC3) or Dulbecco's modified Eagle's medium (DMEM) (βTC6) (Life Technologies, Inc., Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 100 units/ml penicillin and 50 μg/ml streptomycin, and incubated at 37 °C in a humidified incubator with 5% CO_2/95% air (βTC3) or 10% CO_2/90% air (βTC6). Mouse islets were isolated using collagenase as described previously [29] and cultured in RPMI medium containing 11 mM glucose for 1–2 days.

Insulin secretion and cAMP measurement

βTC3 cells were plated in 24-well dishes and used between 50% and 70% confluence. On the day of the experiment they were washed two times with Krebs–Ringer bicarbonate buffer (KRB: 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM Hepes, pH 7.4, and 0.1% BSA) containing no glucose and then preincubated with 1 ml of KRB lacking glucose for 60 min at 37 °C. Medium was removed and cells were incubated for an additional 60 min with 1 ml of fresh KRB containing the tested agents. Incubation buffer was taken for insulin determination by RIA using a rat insulin standard curve and a Miles antibody, and for cAMP assay with a RIA kit from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). Cells were washed with fresh ice-cold KRB, and intracellular cAMP was extracted with perchloric acid and measured as described above. Assays were performed in the RIA core of the Penn Diabetes Center [30].

Measurement of cytosolic free Ca^{2+}

βTC3 cells were plated on coverslips and cultured in RPMI containing 11 mM glucose for 1–2 days. Mouse islets were isolated using collagenase as described previously [31] and cultured in RPMI containing 11 mM glucose for 1–2 days. Cells or islets were then loaded with fura-2 during a 40 min pretreatment at 37 °C in KRB without glucose and with 1 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.). The coverslips with cells were mounted on a Zeiss fluorescence microscope, while the islets were fixed by slight suction on the tip of a micropipette in a chamber placed on the homeothermic platform of the microscope. Cells or islets were incubated in KRB at 37 °C. The microscope was used with a 40× oil-immersion objective. Fura-2 was excited successively at 334 nm and 380 nm by means of two narrow band-pass filters. The emitted fluorescence was filtered through a 520 nm filter, captured with an Attofluor CCD video camera at a resolution of 512 × 480 pixels, digitized into 256 gray levels and analysed with version 6.07 of the Attofluor RatioVision software (Atto Instruments, Rockville, MD, U.S.A.). The concentration of Ca^{2+} was calculated by comparing the ratio of fluorescence at each pixel with an *in vitro* two-point calibration curve. The Ca^{2+} concentration is presented by averaging the values of all pixels in an islet or in a cell. Data points were collected at intervals of 20 s [29].

Western blot analysis of βTC6 cells

βTC6 cells were plated in 10-cm dishes and cultured in DMEM. Cells were serum-starved overnight, and then washed once with glucose-free KRB and incubated with glucose-free KRB for 3 h.

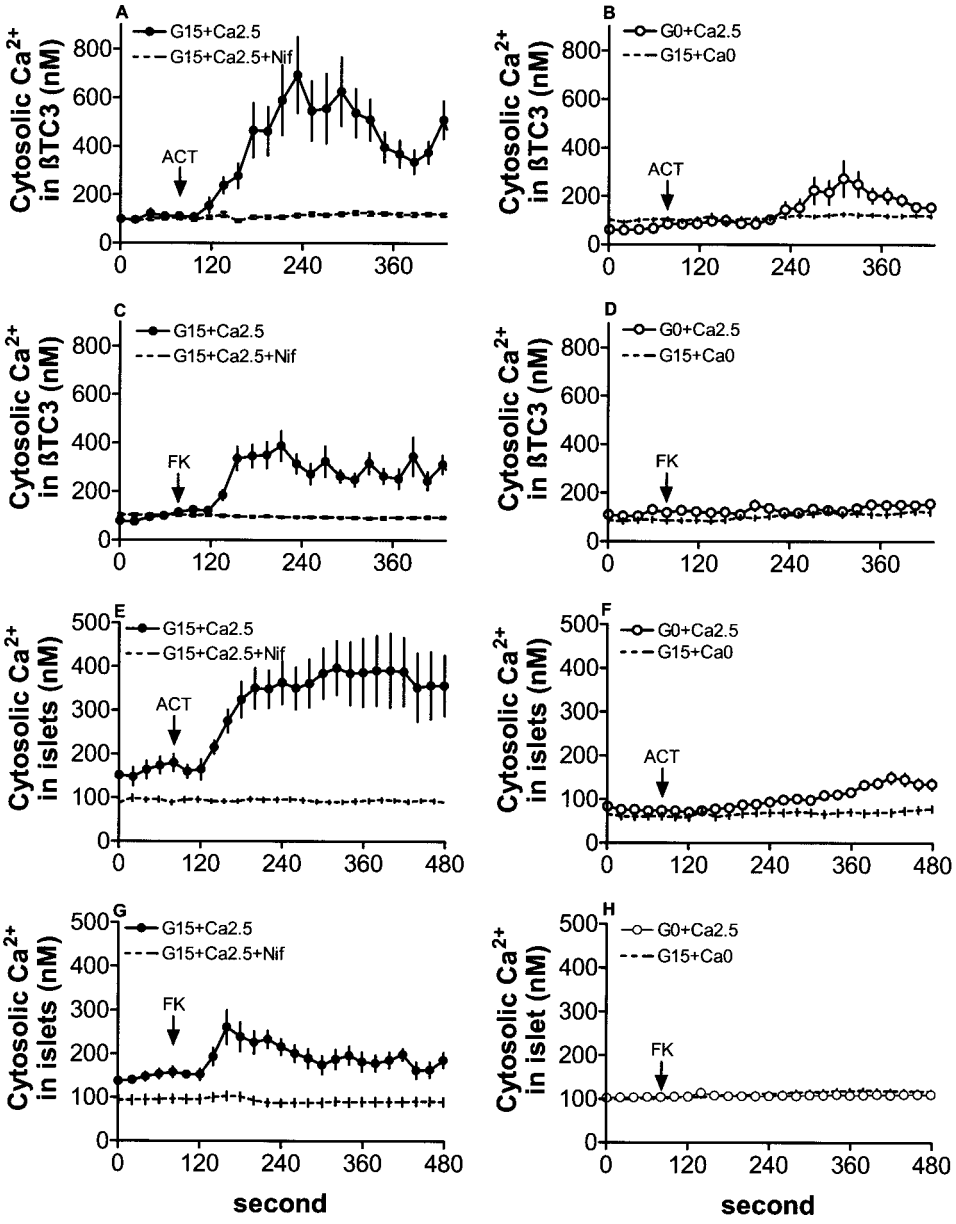

Figure 3 Effects of ACT and forskolin on cytosolic Ca^{2+} levels in βTC3 cells and mouse islets

βTC3 cells were plated on glass coverslips and cultured in RPMI medium containing 11 mM glucose for 1–2 days. Mouse islets were isolated as described in the Experimental section and were also cultured in the presence of 11 mM glucose for 1–2 days. While the Ca^{2+} signal was captured, 1 μg/ml ACT or 10 μM forskolin (FK) was added to the βTC3 cells or mouse islets with four different conditions: 15 mM glucose and 2.5 mM calcium (G15 + Ca2.5), 15 mM glucose, 2.5 mM calcium and nifedipine (G15 + Ca2.5 + Nif), no glucose and 2.5 mM calcium (G0 + Ca2.5) or 15 mM glucose and no calcium (G15 + Ca0). Results are means ± S.E.M. for cytosolic Ca^{2+} from three experiments with 58–59 cells or six islets per condition.

The cells were then incubated in fresh KRB containing various agents. Following incubation they were washed with ice-cold PBS and scraped into 0.3 ml of fresh lysis buffer containing protease inhibitors and phosphatase inhibitors. An equal amount of protein from each incubation was analysed by SDS/PAGE and immunoblotted with an antibody recognizing a specific PKA catalytic subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) or the phosphorylated form of CREB (Upstate Biotechnology, Lake Placid, NY, U.S.A.), followed by detection with ^{125}I-Protein A of high specific radioactivity. The radioactivity in each band was quantified on a PhosphorImager (Molecular Dynamics) using ImageQuant software [32].

Subcellular localization of PKA and CREB in βTC6 cells

Cells were plated on 12-mm glass coverslips and cultured in DMEM. Cells were serum-starved overnight, and then washed once with glucose-free KRB and incubated with glucose-free KRB for 3 h. Cells were then incubated in fresh KRB containing various agents. After this, the cells were washed with ice-cold PBS and fixed with methanol at −20 °C for 15 min. Cells were incubated with rabbit antibodies specific for a PKA catalytic subunit or with rabbit anti-CREB antibodies (Santa Cruz Biotechnology) for 1 h, followed by washing. Fluorescently labelled anti-IgG secondary antibody (Jackson ImmunoResearch

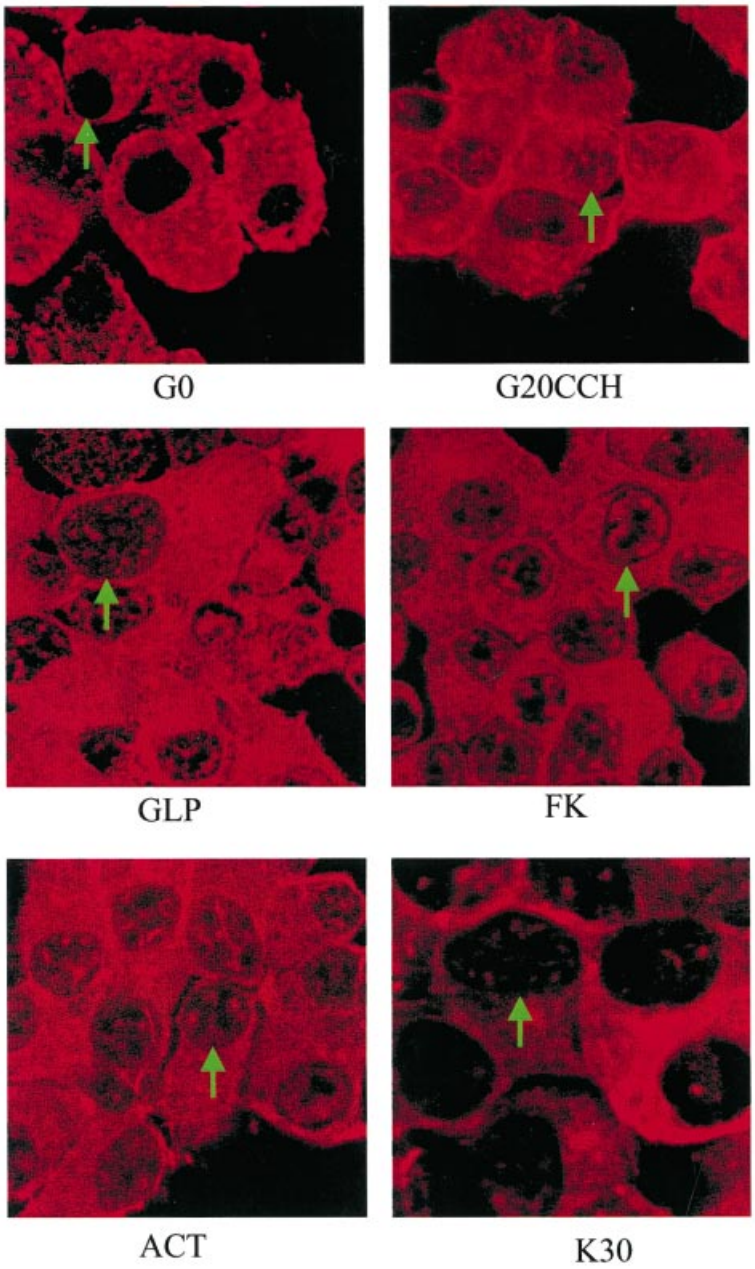

Figure 4 Subcellular localization of PKAα in βTC6 cells

βTC6 cells were plated on 12-mm glass coverslips and cultured in DMEM containing 25 mM glucose for 2–4 days. Cells were serum-starved overnight in the absence of glucose, and then incubated in KRB containing no glucose for 3 h before they were treated with no glucose (G0), 20 mM glucose + 0.5 mM carbachol (G20CCH), 100 nM GLP-1, 10 μM forskolin (FK), 1 μg/ml ACT or 30 mM K^+ (K30) for 40 min. Cells were washed with PBS, fixed in methanol, washed again and stained with primary and Texas Red-conjugated secondary antibodies. Cells were analysed by scanning confocal laser microscopy and transmission microscopy (to identify nuclei; denoted by arrows). Panels show representative scans from three to five experiments.

Laboratories, West Grove, PA, U.S.A.) was subsequently added for 1 h, followed by washing. Coverslips with cells were then fixed on glass slides and the subcellular localization of PKA in cells was examined by laser scan confocal microscopy (Biomedical Imaging Core, DERC, University of Pennsylvania) [33]. Confocal images were then quantified using ImageQuant software.

Data analysis

Student's *t* tests were performed when two groups were compared. ANOVA was used, followed by the Newman–Keuls test, when multiple groups were compared. Differences were considered significant at $P < 0.05$.

Materials

Recombinant ACT was prepared from plasmid pT7ACT1 [34] using calmodulin-affinity chromatography as the final step [26]. The material was eluted and stored in buffer containing urea (8 M), Tricine (10 mM), EDTA (0.5 mM) and EGTA (0.5 mM), pH 8.0, until use, at which time it was diluted at least 100-fold into the medium for that experiment. All other chemicals were

from Sigma (St. Louis, MO, U.S.A.), while molecular biological reagents were from GIBCO BRL (Grand Island, NY, U.S.A.).

RESULTS

ACT stimulates insulin secretion

ACT is a polypeptide produced by *Bordetella pertussis* which enters target cells and generates high levels of cAMP [35,36]. The effects of ACT on insulin secretion were first tested in the presence of 15 mM glucose with concentrations of 0.1–10 μg/ml ACT (Figure 1, upper panel). At a concentration of 10 μg/ml, ACT produced a 2.5-fold increase in glucose-induced insulin secretion ($P < 0.05$ compared with glucose alone). A 1.6-fold increase was produced by 1 μg/ml ACT ($P < 0.05$). On the other hand, ACT did not affect insulin secretion in the absence of glucose at any concentration tested ($P > 0.05$). An unacylated ACT, which is unable to bind and enter target cells, failed to stimulate glucose-induced insulin secretion [37]. The effects of GLP-1 on insulin secretion were also tested in incubated βTC3 cells. Basal insulin secretion was 134±11 ng/well, which was increased by 10 and 100 nM GLP-1 to 240±17 and 229±11 ng/well respectively.

The stimulation of cAMP release by ACT was also assessed. In the presence of 15 mM glucose, ACT dose-dependently increased the release of cAMP from βTC3 cells. The lowest effective concentration of ACT was 1 μg/ml ($P < 0.05$), which resulted in a >100-fold increase in cAMP levels (58.9±11.9 pmol/well) compared with control (0.42±0.05 pmol/well). The slope of the ACT dose–response curve for cAMP release was exponential, with an increase of five orders of magnitude at 10 μg/ml. In the absence of glucose, the basal level of cAMP release was slightly lower than that in the presence of 15 mM glucose. ACT alone also dose-dependently increased cAMP release, but to slightly lower levels than those obtained in the presence of glucose (Figure 1, lower panel).

The influence of extracellular Ca^{2+} on the effects of ACT was tested in the presence of 15 mM glucose (Figure 2). In the presence of 2.5 mM extracellular Ca^{2+} and 15 mM glucose, ACT produced a 2.8-fold increase in insulin secretion (400±14 compared with 141±14 ng/well; $P < 0.05$), and an increase of three orders of magnitude in the intracellular cAMP level ($P < 0.05$). The omission of extracellular Ca^{2+} decreased insulin secretion by 50% ($P < 0.05$), but did not affect the intracellular cAMP level (64±10 compared with 59±6 ng/well; $P > 0.05$). The absence of extracellular Ca^{2+} completely abolished the stimulation by ACT of insulin secretion ($P > 0.05$) and of intracellular cAMP accumulation ($P > 0.05$).

cAMP-dependent regulation of cytosolic Ca^{2+}

The effects of ACT and forskolin on cytosolic Ca^{2+} were studied in βTC3 cells and cultured mouse pancreatic islets (Figure 3). In the presence of 15 mM glucose and 2.5 mM extracellular Ca^{2+}, addition of 1 μg/ml ACT to βTC3 cells caused a rapid and dramatic increase in cytosolic Ca^{2+}, from 113±11 nM to a peak level of 694±156 nM ($P < 0.05$) (Figure 3A). Addition of 1 μM nifedipine at the start of the experiment in the presence of 15 mM glucose did not significantly change the Ca^{2+} level; however, it completely blocked the effects of ACT (Figure 3A). In cultured pancreatic mouse islets, ACT increased cytosolic Ca^{2+} from 165±23 nM to a peak of 391±80 nM ($P < 0.05$) in the presence of 15 mM glucose and 2.5 mM Ca^{2+}. The cytosolic Ca^{2+} level in the presence of 15 mM glucose was decreased by nifedipine, while the effect of ACT was completely blocked when 1 μM nifedipine was added at the start of the experiment (Figure 3E). In the absence of glucose and in the presence of 2.5 mM extracellular Ca^{2+}, ACT produced a much smaller increase in cytosolic Ca^{2+}, from 84±14 nM to a peak of 273±74 nM ($P < 0.05$), in βTC3 cells. When extracellular Ca^{2+} was omitted and 1 mM EGTA and 15 mM glucose were present, ACT had no effect on cytosolic Ca^{2+} ($P > 0.05$) in βTC3 cells. ACT produced similarly small or no effects in mouse islets in the absence of glucose alone or in the absence of extracellular Ca^{2+} alone (Figure 3F).

Forskolin at 10 μM produced an increase in cytosolic Ca^{2+}, smaller than that induced by ACT, in the presence of glucose and 2.5 mM extracellular Ca^{2+}, from 114±15 nM to a peak of 387±62 nM ($P < 0.05$). Nifedipine also abolished forskolin stimulation of cytosolic Ca^{2+} in βTC3 cells when added at the beginning of the experiment (Figure 3C). Similar effects of ACT and blockage by nifedipine were observed in mouse islets (Figure 3G). Forskolin was ineffective either without glucose or without Ca^{2+} alone ($P > 0.05$) in both βTC3 cells and mouse islets (Figures 3D and 3H). In summary, neither ACT nor forskolin increased cytosolic Ca^{2+} in the presence of the Ca^{2+} channel blocker nifedipine. Cytosolic Ca^{2+} was increased by 10 nM GLP-1 to 189±16 nM, which was significantly higher than the value of 106±6 nM under basal conditions.

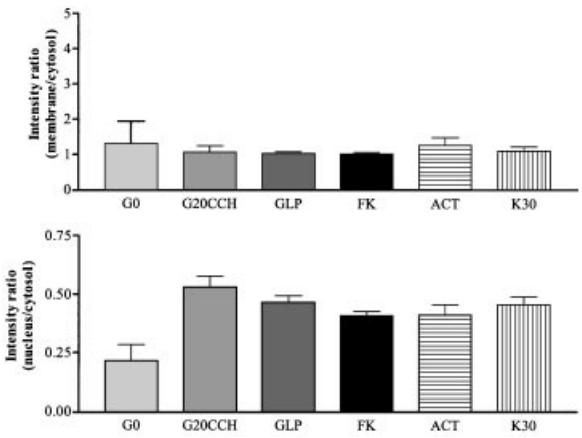

Figure 5 Quantification of the subcellular localization of PKAα in βTC6 cells

Cells were incubated under the following conditions for 40 min: no glucose (G0), 20 mM glucose + 0.5 mM carbachol (G20CCH), 100 nM GLP-1, 10 μM forskolin (FK), 1 μg/ml ACT or 30 mM K^+ (K30). Images acquired as in Figure 4 were analysed with ImageQuant software to determine the fluorescence intensity of three cellular regions: cytosol, plasma membrane and nucleus. Intensities were then normalized by calculating the m/c and n/c ratios. Data are means±S.E.M. of 10–27 cells.

PKA isoforms in insulin-secreting β-cells

The presence of the Cα, Cβ and Cγ catalytic subunits of PKA in βTC6 cells was first demonstrated by Western blot experiments. Their relative amounts were not changed by 1–3 h of treatment with 20 mM glucose + 1 mM carbachol, GLP, forskolin, ACT or 30 mM K^+ (results not shown).

The subcellular localization of PKA catalytic subunits was studied by confocal laser scanning microscopy (Figures 4–7). After overnight serum starvation and a 3 h incubation in the absence of glucose, β-cells were incubated under the various experimental conditions. The cell borders were easily identified by the edges in phase-contrast images and by the low background intensities in fluorescent images. The borders of the nuclei were

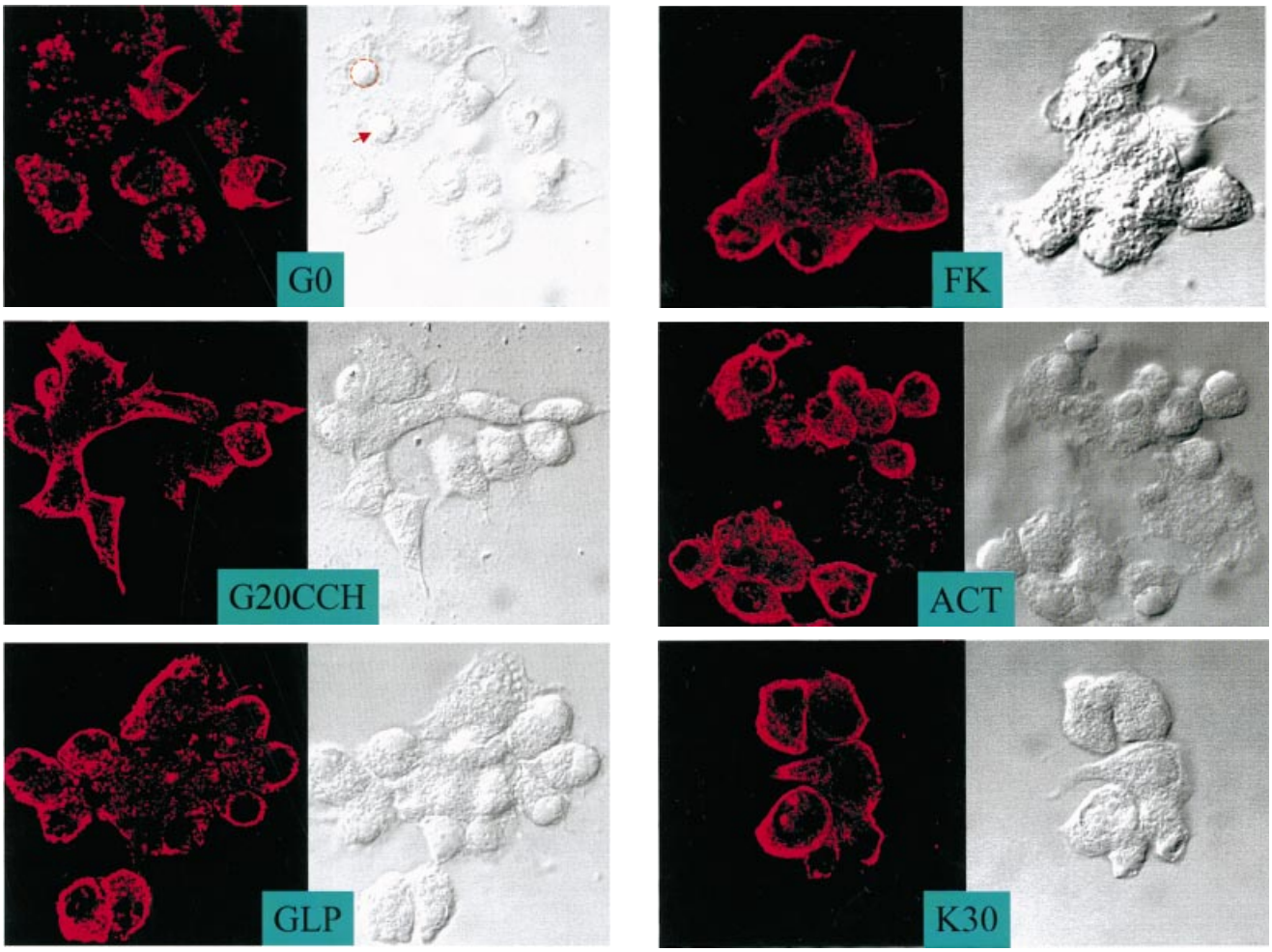

Figure 6 Subcellular localization of PKAβ in βTC6 cells

βTC6 cells were prepared as in Figure 4, except that an antibody to PKAβ was used. Conditions were as follows: no glucose (G0), 20 mM glucose + 0.5 mM carbachol (G20CCH), 100 nM GLP-1, 10 μM forskolin (FK), 1 μg/ml ACT and 30 mM K^+ (K30). Panels show representative scans and transmission micrographs from four experiments.

identified by the distinctive reflection of light in the phase-contrast images, as indicated by a red circle or a red arrow in Figure 6.This is probably due to the elevation of the region above the nuclei. The area inside the cell border but outside the nucleus was considered as the cytosol. For Cα, the nucleus can also be easily identified in the fluorescent image because of its lower intensity of staining. However, the phase-contrast images are needed to identify the nuclei in Cβ images. The fluorescence intensity in three different regions of the cells was quantified using ImageQuant software, and normalized as the membrane/cytosol (m/c) ratio and the nucleus/cytosol (n/c) ratio (Figures 5 and 7).

Under basal conditions (0 mM glucose), PKAα (Figure 4) was located mainly in the cytosolic or extranuclear space, with low nuclear staining, as shown by a very low n/c ratio of 0.22 ± 0.02. The nucleus was identified by confocal scanning pictures (not shown) of the same confocal planes generated with transmitted light. In cells treated with 20 mM glucose plus carbachol (0.53 ± 0.05), GLP-1 (0.47 ± 0.03), forskolin (0.41 ± 0.02) or ACT (0.41 ± 0.05) for 40 min, Cα had a relatively higher nuclear staining ($P < 0.01$), showing that Cα was translocated to the nucleus following treatment with insulin secretagogues. Cells treated with 30 mM K^+ also had higher staining of Cα in the nucleus (0.46 ± 0.03). PKAα staining in the cytosol was not significantly different from that of the control in the absence of glucose. The m/c ratios were all close to 1, indicating that the fluorescence intensity on the membrane was identical with that in the cytosol (Figures 4 and 5).

The subcellular distribution of the Cβ catalytic subunit (Figure 6) was different from that of Cα. Under basal conditions, Cβ staining was found mainly in the cytosol, with minimal staining in the nuclei. When cells were stimulated for 40 min with 20 mM glucose + 1 mM carbachol, GLP-1, forskolin, ACT or 30 mM K^+, the staining on the periphery of cells was much more intense, although some intranuclear translocation of Cβ was also found, as quantified in Figure 7.The fluorescent and light microscopy images were scanned in the middle of most cells, and the cell boundary was thus determined precisely. Under basal conditions (no glucose), membrane staining was about the same as that in cytosol, with an m/c ratio of 0.88 ± 0.16. However, m/c ratios were increased several-fold ($P < 0.01$) following stimulation with 20 mM glucose + 1 mM carbachol (3.10 ± 0.17), GLP-1 (3.69 ± 0.32), forskolin (3.55 ± 0.09), ACT (2.52 ± 0.15) or 30 mM K^+ (2.08 ± 0.015). When the focal plane was moved to the top or the bottom of the cell monolayer, higher Cβ staining was also detected, indicating that it was translocated to the plasma

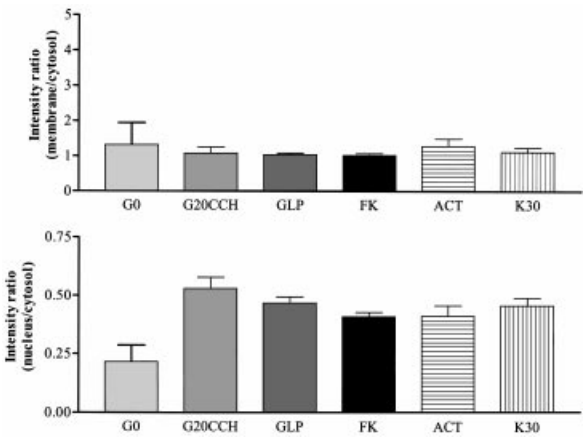

Figure 7 Quantification of the subcellular localization of PKAβ in βTC6 cells

Cells were incubated under the following conditions for 40 min: no glucose (G0), 20 mM glucose + 0.5 mM carbachol (G20CCH), 100 nM GLP-1, 10 μM forskolin (FK), 1 μg/ml ACT or 30 mM K^+ (K30). Images acquired as in Figure 6 were analysed with ImageQuant software to determine the fluorescence intensity of three cellular regions: cytosol, plasma membrane and nucleus. Intensities were then normalized by calculating the m/c and n/c ratios. Data are means ± S.E.M. of 8–31 cells.

membrane and/or its vicinity. The increase in the n/c ratio could be an artifact or could be exaggerated due to the dramatic decrease in intensity in the cytosol (Figure 7, lower panel); however, some intranuclear translocation of Cβ might occur.

Cγ was located in both the cytosol and the nucleus, with an n/c ratio of 0.74 ± 0.02 under basal conditions. Its subcellular location was not changed by the addition of forskolin, 20 mM glucose + carbachol, GLP-1, ACT or 30 mM K^+ for 40 min (results not shown).

DISCUSSION

The presence of PKA in pancreatic β-cells has been known for over a decade, and its involvement in insulin secretion has been proposed in many studies [7,8]. However, the molecular characteristics and downstream signalling of PKA remain poorly understood. We have now identified for the first time the presence of α, β and γ subtypes of the PKA catalytic subunit in pancreatic β-cells. It is likely that each isoform has distinct function(s) in the β-cell, based on its location. The translocation of Cβ from the cytosol to the plasma membrane of the β-cell suggests that substrates of PKAβ are on the plasma membrane. All cAMP-stimulating agents studied (ACT, forskolin and GLP-1), or depolarization, caused translocation of Cβ, but did not always stimulate insulin secretion if glucose was not present (ACT, forskolin and GLP); these findings suggest that Cβ translocation may be the cause of insulin exocytosis, but is not the consequence. Several PKA substrates on the plasma membrane have been identified in other cell systems, including voltage-dependent Ca^{2+} channels in cardiac microsomes [38] and ATP-sensitive K channels transfected into HEK293 cells [39]. Furthermore, in β-cells, phosphorylation of L-type Ca channels by PKA has been demonstrated by another group [40]. It is well established that the L-type Ca^{2+} channel is present in primary pancreatic islet β-cells and insulin-secreting β-cell lines [41]. Further evidence supportive of PKA regulation of Ca^{2+} channels is the observation that GLP-1 causes slower inactivation of the Ca^{2+} current, a prolonged burst of action potential, increased amplitude of the Ca^{2+} current and thus greater Ca^{2+} influx into the pancreatic β-cell [42]. In the present study, activation of PKA by either ACT or forskolin amplified glucose-induced insulin secretion, which is consistent with previous studies. Activation of PKA also increased cytosolic Ca^{2+} levels in βTC3 cells and mouse islets in a glucose- and Ca^{2+}-dependent manner in our present study. This effect was blocked by the L-type Ca^{2+} channel inhibitor nifedipine. Thus our results are compatible with the hypothesis that the L-type Ca^{2+} channel might be a substrate of PKA in β-cells.

Another possible substrate for PKA in β-cells is the K_{ATP} channel. A recent report showed that the ATP-sensitive K^+ channel in HEK293 cells is positively regulated by PKA-mediated phosphorylation of Thr-224 in the Kir6.2 subunit [39], and those authors suggested that PKA phosphorylation of this residue in Kir6.2 may be a general mechanism for regulation of K_{ATP} channels in different tissues. A K_{ATP} channel similar to that studied by Lin et al. [39] is also present in pancreatic β-cells. Both the Kir6.2 and SUR1 subunits of the K_{ATP} channel contain consensus sites for PKA phosphorylation [43,44]. It can be speculated that phosphorylation of K_{ATP} channels by PKA in β-cells could render them more sensitive to signals generated by glucose, such as the ATP/ADP ratio, and could thus potentiate depolarization and Ca^{2+} influx. Consistent with this view is the observation that GLP-1 inhibits K_{ATP} channels in a glucose-dependent manner in isolated rat pancreatic β-cells [45].

Following insulin secretion by the β-cell, insulin stores in the secretory granules need to be replenished. This requires regulated transcription of preproinsulin mRNA and translation of the corresponding protein. Physiological stimuli such as glucose [46] and GLP [47] are known to increase insulin mRNA levels in β-cells. The Cα and Cβ subunits are translocated to the nuclei and may trigger insulin transcription. The role of Cγ is still unclear, since it has a relatively high intranuclear level under basal conditions and is not translocated after stimulation. However, further studies in other β-cell types and primary islet β-cells will be needed, since there might be differences between transformed cell lines and primary cells. The possibility cannot be excluded that the distribution and/or functions of PKA catalytic subunits vary between βTC6 cells and primary β-cells. Although cAMP release is increased by ACT or forskolin, the physiological significance of this is currently unknown.

It has been suggested that isoenzymes of PKA with specific subcellular locations mediate distinct effects of cAMP, which are determined by the regulatory subunit (type I or type II). However, the type I PKA isoenzymes are generally soluble and cytosolic ([48]; see also [48a]), which would allow them to regulate all activities in the cytoplasm that are triggered by cAMP. Our study has demonstrated a novel mechanism of isoenzyme (Cα versus Cβ) specificity via distinct patterns of translocation (nucleus versus plasma membrane), which are determined by the catalytic subunits instead of the regulatory subunits. The high content of intranuclear PKAγ under both basal and stimulated conditions is in agreement with a previous report [48,48a].

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