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cAMP-Enhancing Agents "Permit" Stimulus-Secretion Coupling in Canine Pancreatic Islet β-Cells

D.W. Barnett, D.M. Pressel, H.T. Chern, D.W. Scharp, S. Misler

Departments of Medicine, Cell Biology/Physiology and Surgery, Washington University Medical Center, St. Louis, Missouri 63110

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Abstract. Isolated canine islets of Langerhans differ from isolated islets of other species (including rodents and man) in that elevated glucose concentrations are unable to stimulate insulin secretion. Here we demonstrate that addition to the perifusate of isobutylmethylxanthine (IBMX), forskolin or 8-CPT-cAMP, all of which enhance cytosolic cAMP, *permits* insulin secretion in response to glucose, leucine or tolbutamide. These cAMP enhancers increase secretogogue-induced electrical activity in β-cells and restore depolarization-induced, Ca^{2+}-dependent granule exocytosis measured as stepwise increases in membrane capacitance. We propose that the primary permissive action of cAMP is to tightly link Ca^{2+} entry to insulin granule release, while a secondary action is to tighten the link between glucose metabolism and cell depolarization.

Key Words: Cyclic AMP — Insulin secretion — Electrical activity — Ca^{2+} currents — Membrane capacitance

Introduction

The role of cyclic adenosine-5′-monophosphate (cAMP) in glucose-induced insulin release by β-cells of pancreatic islets of Langerhans is complex. In whole rodent islets, where these effects have been investigated most intensively, cAMP levels rise during glucose stimulation (Grill & Cerasi, 1974), but inhibition of adenylate cyclase or cAMP-dependent protein kinase does not affect insulin secretion in a consistent manner (Persaud, Jones & Howell, 1990). However, addition to the bath of agents which raise cAMP concentration greatly enhances electrical activity and insulin secretion induced by glucose or other secretogogues (*see* Henquin, 1985, for review). On the other hand, in isolated rodent β-cells, glucose often fails to induce membrane depolarization, elevation of cytosolic Ca^{2+} and insulin secretion in the absence of a cAMP-enhancing agent (e.g., Pipeleers et al., 1985; and Holz, Kühntrieber & Habener, 1993; Wang et al., 1993).

Recently, Scharp et al. (1989) reported that newly harvested dog islets failed to secrete insulin in response to stimulation by glucose or leucine. Secretion was restored by addition to the perifusion solution of a phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) at 1 mM and carbamylcholine. Here we report that IBMX is sufficient to permit glucose- or tolbutamide-induced insulin secretion in whole islets. The effect of IBMX is mimicked by a direct activator of adenylate cyclase (forskolin) and a membrane permeant cAMP analogue (8-CPT cAMP), suggesting that all of these agents permit secretion by enhancing cytosolic cAMP. In the absence of cAMP enhancers, β-cells responded to 20–40 μM tolbutamide with vigorous electrical activity and increments in cytosolic Ca^{2+} but often failed to respond to 15 mM glucose until a cAMP enhancer was present. Additionally, in the absence of a cAMP enhancer, a majority of islet cells failed to show depolarization-induced increases in membrane capacitance (ΔC_m) associated with β-cell granule exocytosis, despite having sizeable Ca^{2+} currents. ΔC_m's were restored after addition of forskolin and/or IBMX. These results suggest that Ca^{2+} enhancers *primarily* serve to restore insulin secretion by tightly linking Ca^{2+} entry to the process of insulin granule release, and *secondarily* to enhance secretion by tightening the link between glucose metabolism and depolarization.

Correspondence to: S. Misler, Renal Division (Yalem 815), Jewish Hospital of St. Louis, 216 S. Kingshighway Blvd., St. Louis, Missouri 63110

Materials and Methods

1. Islet Preparation and Characterization

The islets used in this study were prepared by the Islet Transplantation Laboratory, Washington University. Briefly, pancreata were harvested from mongrel dogs. Islets were isolated using an automated protocol (Olack et al., 1992) nearly identical to that previously described for human cadaver pancreases (Ricordi et al., 1988).

The following pilot experiments were carried out to investigate the structural and metabolic integrity of freshly isolated canine islets. (1) Aliquots of islets from three different preparations were fixed with formaldehyde and stained with osmium tetraoxide, and later thin-sectioned for electron microscopy. Many sections revealed cells with dense core granules, typical of A and D cells, adjacent to cells with crystalline-filled granules, typical of β-cells. In rare sections, capillary endothelial cells were discernable (S. Misler, D. Pressel and R. Henry, *unpublished data*). (2) Aliquots of islets from two different preparations were exposed to ^{14}C-labeled glucose. These islets demonstrated a threefold increase in $^{14}CO_2$ production in the presence of 16.8 *vs.* 3 mM glucose. The presence of IBMX did not alter baseline or stimulated $^{14}CO_2$ production (D. Pressel and S. Misler, *unpublished data*). (3) Lastly, aliquots of islets from two preparations were exposed to elevated K^+ and assayed for glucagon secretion by radioimmunoassay. In the presence of 1 mM IBMX, raising the K^+ concentration of the bath from 5.5 to 30 mM increased glucagon secretion by fourfold (D. Barnett and S. Misler, *unpublished data*).

2. Measurement of Insulin Secretion

Insulin secretion from isolated perifused islets was measured by radioimmunoassay, as previously described (Pressel & Misler, 1991). After isolation and purification, islets were cultured for 1–3 days at 37°C. Equal aliquots of the islet cell suspensions usually containing 75–100 islets were loaded into paired chambers containing a cellulose acetate filter (8 μm) (Millipore, Bedford, MA). The chambers, incubated in a 37°C water bath, were perifused with an oxygenated solution (95% O_2/5% CO_2) containing (in mM): 115 NaCl, 24 $NaHCO_3$, 5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 0.5% RIA grade albumin. The flow rate of the perifusate was ~1 ml/min and effluent from each islet-containing chamber was collected manually at intervals from 1–5 min. The samples were frozen and, subsequently, insulin was measured by the Diabetes Research and Training Center Radioimmunoassay Core Facility at our institution. Data on time course of insulin secretion presented in Fig. 1 are representative of those obtained from duplicate perifusion experiments performed on at least three different islet isolates.

3. Recording of Electrical Activity and Membrane Conductance of Islet Cells

The methods used to record these parameters from islets were nearly identical to those described in a prior study of canine β-cells (Pressel & Misler, 1991). Islet clumps were bathed in a standard extracellular solution containing (in mM): 144 NaCl, 5.5 KCl, 2.0 $CaCl_2$, 1 $MgCl_2$, and 20 HEPES titrated to pH 7.3 with NaOH. Individual islet cells or cells in small clumps were recorded from using the perforated patch variant of whole cell recording. Glass micropipettes were filled by immersion of the tip in a high K^+ buffer solution which contained (in mM): 67.5 KCl, 28.4 K_2SO_4, 11.8 NaCl, 1 $MgCl_2$, 0.5 EGTA, 47.2 sucrose, and 20 HEPES titrated to pH 7.3 with KOH. They were then backfilled with a high K^+ buffer solution which contained, in addition, 150–300 μg/ml nystatin (Sigma, St. Louis, MO). Action potential activity was recorded in the current clamp mode using either the EPC-7 or the EPC-9 patch clamp amplifier (Heka, Darmstadt, Germany), at room temperature (21–23°C), to enhance the mechanical stability of the patches. Background membrane K^+ conductance was assessed by applying voltage clamp pulses which hyperpolarized the membrane by 20–30 mV from rest and then measuring the evoked current. Data collection was begun once the access resistance (R_a) from the pipette and the cell interior fell to less than 100 MΩ. Cells were identified physiologically as β-cells by the presence of ATP-sensitive K^+ channels in the cell-attached patch prior to patch perforation or whole cell ATP-sensitive K^+ currents after patch perforation (*see* Figs. 3 and 4, Pressel & Misler, 1991).

4. Microspectrofluorimetry for Cytosolic Ca^{2+} Determination

The methods used for these determinations were nearly identical to those we previously adopted for human islets (Misler et al., 1992). Glass coverslips dotted with islets were incubated for ~60 min at room temperature in the dark in culture medium containing 2 μM Fura 2-AM (Molecular Probes, Junction City, OR) previously dissolved in dimethylsulfoxide (DMSO). The coverslips were rinsed and mounted in a recording chamber on the stage of an inverted microscope in the light path of a Spex Fluorolog Cation Measurement System. Uniformly fluorescent islet cell clumps, viewed at 400 or 1,000×, were chosen for monitoring at 30–32°C. Fluorescence emission (F) at 505 nm was measured in response to rapid alternate excitation of the trapped intracellular Fura 2-AM at 340 and 380 nm, and was displayed as a running fluorescence ratio R (F_{340}/F_{380}). These experiments proved difficult with only 40% (6/16) of cell clumps responding to secretogogues, even under optimal conditions. The amount of Fura 2-AM used optimized the dye loading while minimizing the visual evidence of cell injury (i.e., the development of membrane blebbing) during 20–30 min of light exposure.

5. Depolarization-induced Changes in Membrane Capacitance

Methods used for these determinations were nearly identical to those we previously described for rat β-cells (Gillis & Misler, 1992, 1993). Single islet cells (>10 μm in diameter and membrane capacitance >4 pF) were selected for perforated patch recording in the voltage clamp mode at 30–32°C. The tip of the pipette was filled with a high Cs^+ solution containing (in mM): 28 Cs_2SO_4; 64 CsCl; 12 NaCl; 0.5 EGTA; 1 $MgCl_2$; and 20 HEPES titrated to 7.3 pH with TEAOH. The pipette was then backfilled with the high Cs^+ solution containing 500–1,000 μg/ml nystatin and 0.05–0.2% DMSO. The bath solution (TEA and TTX-modified extracellular solution) contained (in mM): 85 NaCl; 50 tetraethylammonium (TEA) chloride; 5.5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 5 glucose; 5–10 × 10^{-4} tetrodotoxin (TTX); and 20 HEPES titrated to pH 7.3–7.4 with NaOH. To enhance Ca^{2+} currents and Ca^{2+}-dependent release, in some experiments, the Ca^{2+} concentration of the bath was raised to 11.8 mM and BAYK 8644 (2.5 μM) was added. Stimulus-evoked changes in ΔC_m were measured using a two phase lock-in amplifier (LIA) custom designed by K.D. Gillis in conjunction with an EPC-9 patch-clamp amplifier (PCA). After capacity transient neutralization, a 30 mV peak-to-peak sine wave (977 kHz), riding on a −70 mV offset, was applied as a stimulus. The output of the LIA was monitored at a phase angle (α) sensitive to C and at a phase angle orthogonal to α (i.e., α-90°) to track G, a composite of the R_a and membrane conductance. α was determined by

adjusting the phase control of the LIA until the G output was insensitive to offsetting the C_{slow} compensation of the PCA by 0.1 pF. The displacement of the C output of the LIA was used to calibrate the magnitude of depolarization-induced ΔC_m's. Slow changes in patch permeabilization necessitated periodic updating of capacity transient compensation as well as redetermination of α. Due to the data acquisition algorithm used, the C trace is blanked out for one sec after imposition of the depolarizing pulse.

Results

This study was prompted by a previous finding that perifused canine islets, which do not secrete measurable amounts of insulin in response to glucose (6–20 mM) or an alternative metabolite secretogogue leucine, show rapid onset of biphasic secretion following addition of 1 mM IBMX and 10 μM carbachol (Scharp et al., 1989). Figure 1*a* demonstrates that addition of 1 mM IBMX is sufficient to restore insulin secretion in response to 15 mM glucose. Similarly, IBMX restored insulin secretion in response to 40 μM tolbutamide (*b*). The permissive action of IBMX on glucose-induced insulin secretion can be mimicked either by (i) forskolin, but not by its inactive derivative dideoxyforskolin, which lacks cyclase-activating activity (*c*), or (ii) the membrane permeant of cAMP analogue 8-CPT cAMP (*d*). In two pilot experiments, addition of 50 μM Rp-cAMP, an optical isomer of cAMP, which binds, but does not activate, protein kinase A, reduced glucose-induced insulin secretion in the presence of 10 μM forskolin by >50%. These results strongly suggest that IBMX, forskolin and 8-CPT-cAMP all affect insulin secretion by their abilities to raise cAMP. In the presence of a cAMP enhancer, glucose-induced insulin secretion from canine islets was dependent on adequate extracellular Ca^{2+} concentration and intact oxidative metabolism. Glucose-stimulated insulin secretion was reversibly abolished by reducing the extracellular Ca^{2+} concentration from 2 to 0.05–0.1 mM or by adding 3 mM sodium azide to the bath (*data not shown*).

In rat and mouse islets, where cAMP enhancers augment but are not obligatory for nutrient- or tolbutamide-induced secretion, cAMP enhancers have been reported to increase secretogogue-induced electrical activity, Ca^{2+} entry, and depolarization-induced insulin granule exocytosis. To define potential loci of action of cAMP in dog islets, we performed patch clamp electrophysiology to evaluate single cell electrical activity and exocytosis as well as microspectrofluorimetry to evaluate cytosolic Ca^{2+} concentration.

In perforated patch experiments performed under current clamp conditions >85% of all islet cells tested depolarized in response to the application of 20–40 μM tolbutamide in 3 mM glucose. Figure 2 presents the range of glucose-stimulated electrical activity in tolbutamide-sensitive cells. Panel *A* depicts a β-cell, typical of 7/18, which maintained a resting potential of nearly −60 mV in the presence of 15 mM glucose and showed no electrical activity until shortly after the addition of 1 mM IBMX. Thereafter, cells depolarized to −40 mV and conducted a train of large amplitude action potentials which then degenerated to a plateau-like depolarization at −25 mV. Panels *B* and *C* display cells typical of the remaining 11 cells, which slowly depolarized and developed spiking activity on increasing glucose from 3 to 15 mM. However, the cell's rate of depolarization and intensity of spiking activity was greatly enhanced on re-exposure to 15 mM glucose in the presence of either 1 mM IBMX or 10 μM forskolin. Visual inspection of the morphology of the earliest individual action potentials provoked in the presence and absence of a cAMP-enhancing agent revealed no obvious change in action potential threshold, upstroke velocity or overshoot, or duration parameters which would be indicative of gross changes in Na^+ channel activation or inactivation, or K^+ channel activation.

In companion voltage clamp experiments (*see* Fig. 3), we demonstrated clearly that IBMX (0.1–1.5 mM) or forskolin (10–30 μM), in a concentration-dependent manner, reducing resting membrane conductance calculated from the current flow (I_m) evoked by hyperpolarizing the membrane from a holding potential of −60 mV to a clamping potential of −80 mV at a constant glucose concentration (2 mM). The bath concentration of IBMX needed to reduce I_m by half is ~300 μM (*see* Fig. 3). We have previously shown that this current is increased by bath application of sodium azide but is rapidly reduced by tolbutamide (20–40 μM), even in the absence of IBMX or forskolin (*see* Fig. 4, Pressel and Misler, 1991). These features suggest that this current is carried by metabolically regulated, sulfonylurea-inhibited K^+ (ATP) channels.

Panels *a* and *b* of Fig. 4, which depict two of the set of six successful experiments, demonstrate that exposure to 20–40 μM of tolbutamide consistently increases cytosolic Ca^{2+}, even in the absence of cAMP-enhancing maneuvers, while 15 mM glucose did so only inconsistently (i.e., in 2/6 cell clumps) unless 1 mM IBMX is present. Note that in the presence of IBMX, cytosolic Ca^{2+} is enhanced by the dihydropyridine Ca^{2+} channel agonist BAYK 8644 and depressed by reducing extracellular Ca^{2+}, confirming the dependence of the increase in cytosolic Ca^{2+} on Ca^{2+} entry.

The ability of a cAMP enhancer to effectively couple depolarization and Ca^{2+} entry to secretion was further tested by combining phase detection techniques with perforated patch recording in the voltage clamp mode. In this approach, exocytosis of cell granules is measured as the increase in ΔC_m in response to cell depolarization and evoked Ca^{2+} current (Gillis & Misler, 1992, 1993, Ämmälä, Ashcroft & Rorsman, 1993). In rodent β-cells, depolarization-induced ΔC_m's resemble

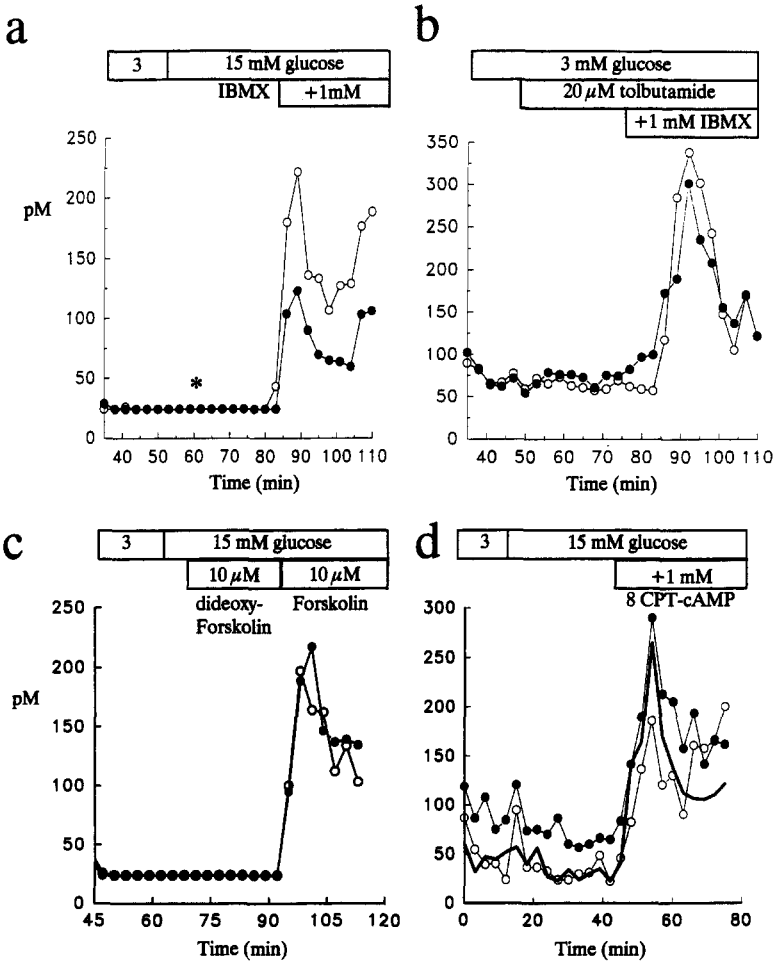

Fig. 1. IBMX, forskolin and 8-CPT-cAMP permit secretogogue-induced insulin secretion. (*a*) Duplicate experiment, out of six, demonstrating that 1 mM IBMX restores glucose-induced insulin secretion. Asterisk over values <25 pM indicates that values were below the lower limits of the assay. (*b*) Duplicate experiment, out of four, demonstrating that 1 mM IBMX permits tolbutamide-induced secretion. (*c*) Paired experiment, out of three, demonstrating that forskolin (10 μM), but not dideoxyforskolin, permits glucose-induced insulin release. (*d*) Paired experiment, out of three, demonstrating that 1 mM 8-CPT-cAMP permits glucose-induced insulin release as well as 1 mM IBMX (unbroken line).

secretogogue-induced insulin release: both are highly dependent on Ca^{2+} entry, being blocked by reduced extracellular Ca^{2+} or addition of a heavy metal Ca^{2+} channel blocker such as cadmium; and both show similar temperature dependence, being blocked at <27°C. Furthermore, depolarization-induced increases in ΔC_m show similar voltage dependence as the immediately preceding Ca^{2+} currents. The smallest ΔC_m's detected (~1.5 fF) are very nearly the size predicted for a 250 nm diameter spherical granule (K. Gillis and S. Misler, *unpublished data*). These findings suggest that depolarization-induced ΔC_m's may adequately reflect insulin granule exocytosis.

In 9/12 canine β-cells tested, voltage clamp pulses from −80 mV to 0 or + 10 mV, V_c's which approach the value of the overshoot of the action potential and evoke peak Ca^{2+} currents, produced no discernable increases in membrane capacitance, until forskolin (10 μM) was added to the bath. The depolarization-induced increases in membrane capacitance seen after addition of forskolin occurred in the absence of consistent increases in peak Ca^{2+} current or the total charge carried by Ca^{2+} current evoked at a given V_c, or a shift in the voltage dependence of the current (*see* Fig. 5).

Discussion

Early studies with isolated canine islets of Langerhans presented an interesting quandary. While isolated islets failed to secrete insulin in response to in vitro perifusion with even massively elevated levels of glucose (Scharp et al., 1989), they appeared to be structurally and metabolically intact, and they secreted quite well in vivo. When injected into the circulation of the spleen or liver of pancreatectomized dogs (Kneteman, Alderson & Scharp, 1987), freshly isolated islets served to normalize serum glucose levels measured in response to mixed meal-oral glucose tolerance tests and prevent ketoacidosis. Here we demonstrate that agents augmenting cytosolic cAMP suffice to restore, in vitro, glucose or tolbutamide-regulated insulin secretion and present evidence implicating two regions in the cascade of stimulus-secretion coupling which are targets of cAMP action. Since many hormones such as glucagon and glucagon-like intestinal peptide increase cAMP, they may be the source of cAMP enhancement in the intact organism or the transplant recipient.

Our most revealing new observation is that while exposure to tolbutamide or glyburide, in the absence of

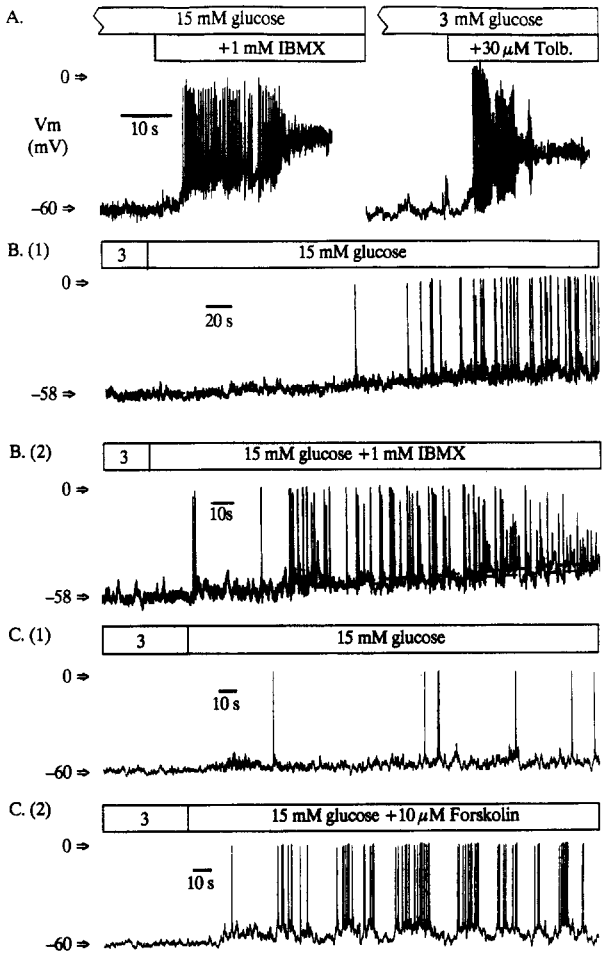

Fig. 2. Effects of IBMX and forskolin on glucose-induced β-cell depolarization and electrical activity. (*A*) Current clamp recording from a cell which maintained a resting potential near −60 mV despite prolonged exposure to 15 mM glucose, but rapidly depolarized and fired action potentials which deteriorated to plateau depolarization after addition of 1 mM IBMX. After washout of IBMX, and return of membrane potential to near −60 mV, addition of 30 μM tolbutamide, now in 3 mM glucose, again resulted in rapid depolarization and the onset of spiking activity. (*B*) Current clamp recording from a cell in which an increase in glucose from 3 to 15 mM resulted in an initial slow depolarization at the onset of spiking activity (Trace *B1*). Electrical activity subsided with a return to 3 mM glucose. Subsequent reexposure to 15 mM glucose in the presence of 1 mM IBMX resulted in at least a twofold higher rate of depolarization (Trace *B2*). Note the time scale change between these two traces. (*C*) Sample traces from a cell in which the change from 3 to 15 mM glucose resulted in a slow depolarization with rare spiking activity, in the absence of forskolin, but impressive spiking in its presence.

cAMP-enhancing agents, invariably results in cell depolarization, electrical activity and a rise in cytosolic Ca^{2+}, it does not result in insulin secretion. Instead, the addition of a cAMP-enhancing agent is critical for the consistent onset of sulfonylurea-induced insulin secretion. The simplest interpretation of these observations is that, on a population basis, the *permissive* effect of

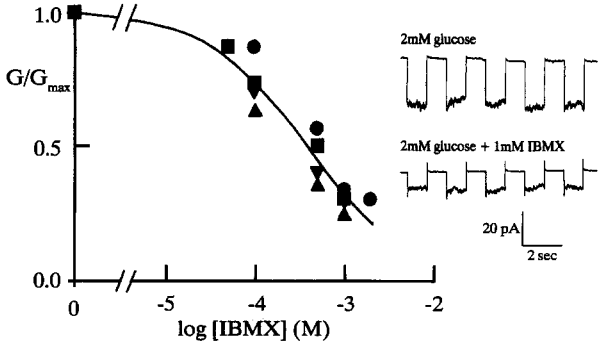

Fig. 3. Dose-dependent decrement of background K^+ conductance (G/G_{max}) in β-cells resulting from bath-applied IBMX. The four different symbols indicate results obtained in separate experiments. The inset depicts sample current traces in response to repetitive voltage clamp pulses from −60 to −80 mV.

cAMP-enhancing agents *primarily* results from the ability of these agents to link electrical activity and a rise in cytosolic Ca^{2+} to insulin secretion, which is otherwise largely dissociated. This interpretation is bolstered by the finding that only a minority of isolated β-cells displays depolarization-induced increases in membrane capacitance, attributable to insulin granule exocytosis, unless a cAMP-enhancing agent is present. However, the ability of these agents to enhance glucose-induced depolarization and electrical activity suggest that cAMP enhancers exert a secondary effect in coupling cell metabolism to depolarization.

Our results complement previously published studies in isolated rat β-cells on the requirement for enhanced cytosolic cAMP in restoring glucose-induced insulin secretion and extend the range of possible mechanisms of action of cAMP in restoring secretogogue-induced insulin release. Studies on isolated rat β-cells suggest that, in the native state, β-cells require some background activation of cAMP-dependent protein kinase A. Using single β-cells purified by fluorescence-activated cell sorting, Wang et al. (1993) correlated the failure of glucose-induced insulin release with the often poor increase in cytosolic Ca^{2+} in β-cells in the absence of cAMP enhancers. Noting that theophylline or glucagon restored insulin secretion as much as it enhanced Ca^{2+} response on poorly responsive cells, they suggested that cAMP enhancement might restore or enhance Ca^{2+} currents in these cells. Holz et al. (1993) correlated the previously reported poor insulin release from single islet cells with poor glucose-induced depolarization and electrical activity, and also found that addition of cAMP enhancers increased closure of K^+ (ATP) channels. Neither of the earlier studies focused on the possibility that a cAMP-enhancer might act to restore secretion, largely by enhancing a Ca^{2+}-dependent feature of the secretory process. Recent studies measuring increases in membrane capacitance in patch-

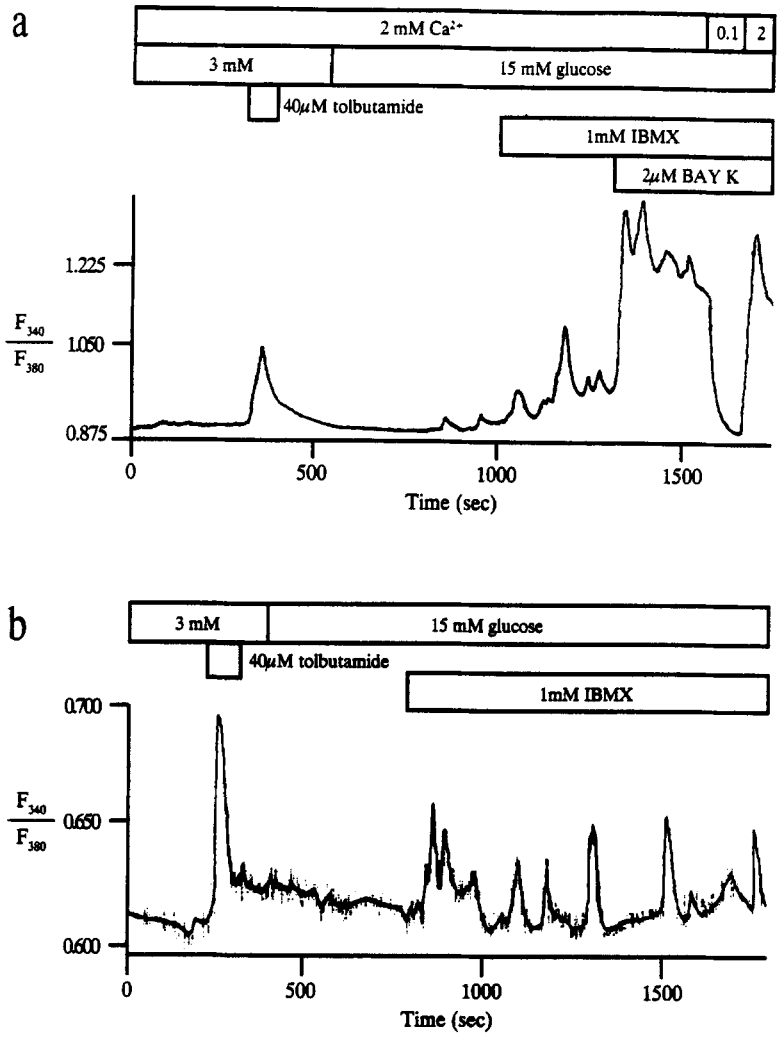

Fig. 4. Glucose- and tolbutamide-induced rises in cytosolic Ca^{2+} in Fura 2-AM loaded canine islet fragments. The increasing value of the running fluorescence ratio R (F_{340}/F_{380}) indicates rising cytosolic Ca^{2+} levels. Note that in the islet fragments depicted in panels *a* and *b*, addition of 30 μM tolbutamide to 3 mM glucose produced a sizable rise in fluorescence ratio, while increasing glucose to 15 mM produced little or no effect until 1 mM IBMX was added. Note in panel *a* that in the presence of IBMX, cytosolic Ca^{2+} rose dramatically with further addition of 2 μM BAYK, a Ca^{2+} channel opener, and reversibly dropped to baseline levels on reducing extracellular Ca^{2+} to 0.1 mM. Trace in panel *b* was darkened for emphasis.

clamped rodent islet cells or insulin secretion from permeabilized islets (Jones, Fyles and Howell, 1986) point towards direct enhancement of the secretory process by cAMP. It is worth noting that, in our experience (Gillis & Misler, 1993), rodent islet cells, prepared and cultured in a fashion similar to the canine cells, are more likely to exhibit depolarization-induced exocytosis in the absence of a cAMP enhancer (i.e., 8/9 rat cells *vs.* 2/10 canine cells), despite comparable Ca^{2+} currents.

Our data do not address two important questions. (i) Why is insulin secretion by isolated dog islets so unresponsive to glucose unless enhancers of cAMP are utilized? (ii) Does cAMP enhancement actually *permit* stimulus-secretion coupling in vivo and, if so, what agent is responsible for cAMP enhancement? Put otherwise, do canine β-cells have a higher turnover rate of cAMP or require higher levels of cAMP for secretory competence from those of other species? Is the cAMP content of canine β-cells more dependent on long-range endocrine interactions (e.g., intestinal secretion of glucagon-like peptide) than on short range paracrine interactions (e.g., local secretion of glucagon or cell-cell coupling) thought to play a crucial role in cAMP enhancement in rodent β-cells (e.g., Pipeleers et al., 1985)? More direct answers to these questions will require cAMP measurements as well as assessment of the intactness of cell-to-cell communication in dog islets in vitro. Several lines of evidence, however, argue against wholesale disruption of islet function or loss of cell surface receptors during isolation as a cause for their unusual physiology. These include: (i) structural and functional evidence for intactness of islets presented above in Section 1 of Materials and Methods; (ii) the rapidity of restoration of stimulus-secretion coupling on exposure to cAMP enhancers; and (iii) preliminary evidence that glucagon (50–100 nM) also permits glucose-induced secretion (D. Barnett and S. Misler, *unpublished data*). It is unclear whether the cAMP requirement extends to other secretogogues. For example, there is evidence that some batches of islets which fail to respond to glucose or tolbutamide in the absence of

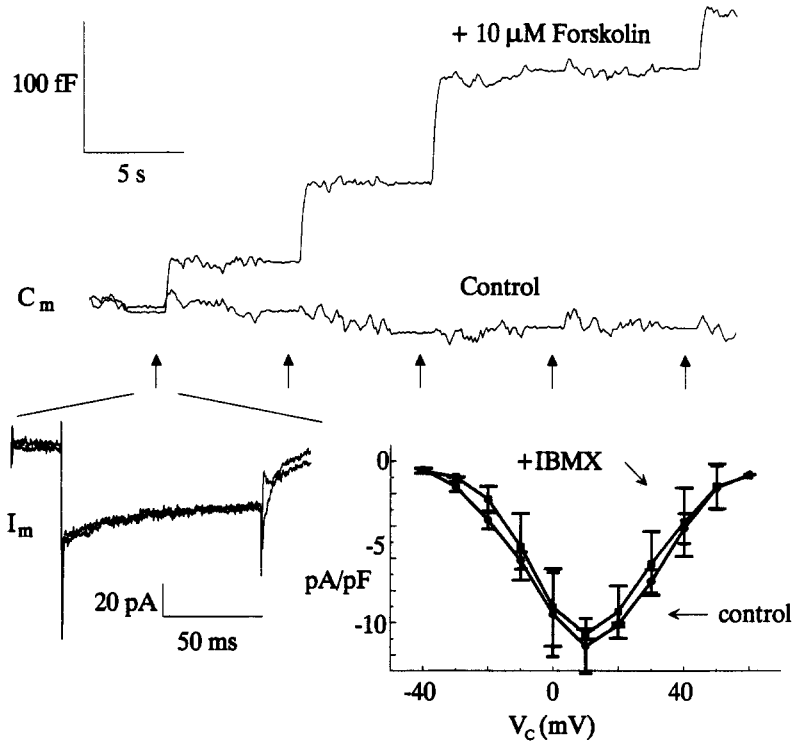

Fig. 5. Forskolin "permits" Ca^{2+}-dependent depolarization-induced increases in cell membrane capacitance in islet cells. Note that step changes in membrane capacitance in response to repetitive 100 msec depolarizations to 0 mV (applied at arrows) are discernable only after addition of 10 μM forskolin to a TEA and TTX-modified extracellular bath solution. Pulses 1-3 and pulse 5 each evoked sustained changes in membrane capacitance of at least 40 fF (assumed to correspond to the exocytosis of at least 20–25 granules). Note the absence of augmentation of inward membrane current after the addition of forskolin. The transient component is a residual Na^+ current not blocked by TTX and partial Na^+ replacement, while the more slowly inactivating component is the high voltage activated (or HVA) Ca^{2+} current. The inset shows that the current/voltage relationship of whole cell Ca^{2+} currents is not significantly altered by the addition of 1 mM IBMX to the bath (N = 3).

IBMX secrete insulin during exposure to 20-30 mM KCl (D. Pressel and S. Misler, *unpublished data*). However, in rodent islets, prolonged entry of Ca^{2+} during KCl stimulation raises cAMP levels (Wang et al., 1993).

Our experiments pose several interesting questions which shall be the subject of further studies. (i) *How do cAMP-enhancing maneuvers serve to strengthen the link between the rise in Ca^{2+} and insulin granule exocytosis?* In many secretory systems, it has been proposed that some degree of cAMP-dependent phosphorylation of plasma membrane or vesicle membrane protein may be needed to permit Ca^{2+}-mediated fusion of the granule with the plasma membrane, either through the release of granules from restraining microfilaments or depolymerization of a cortical actin barrier interposed between the granule and the plasma membrane (Burgoyne, 1991). Some direct support for this hypothesis is provided by membrane capacitance measurements which demonstrate that exocytosis can be increased by cAMP, even in the absence of enhanced Ca^{2+} entry or rise in average cytosolic Ca^{2+} (Ämmälä et al., 1993; Gillis & Misler, 1993).

(ii) *How do cAMP-enhancing agents enhance glucose-induced depolarization?* Our experiments support the hypothesis that the enhancement of glucose-induced depolarization is accompanied by a decrease in membrane conductance, likely due to the closure of K^+ (ATP) channels. In preliminary cell-attached patch experiments, performed on β-cells exposed to a low glucose bath, addition of IBMX produced rapid and reversible closure of single K^+ (ATP) channels (D. Pressel and S. Misler, *unpublished data*). As yet, it is not clear whether cAMP directly affects channel gating or whether it alters β-cell intermediary metabolism, for example by stimulating glycolysis or even glycogenolysis. As noted in Section 1 of Materials and Methods, the $^{14}CO_2$ production from ^{14}C-labeled glucose is not enhanced by 1 mM IBMX. However, stimulation of glycogenolysis might not enhance $^{14}CO_2$ production.

In conclusion, in our early experiments with isolated canine islets, we noted two features not seen with rodent islets typically used in in vitro studies of stimulus-secretion coupling in β-cells. First, whole perifused canine islets, despite apparently intact islet structure, failed to secrete insulin in response to even supraphysiological glucose concentrations unless other *permissive* factors (e.g., IBMX or IBMX and carbamylcholine) were present (Scharp et al., 1989). Second, canine β-cells displayed a unique electrical activity pattern (Pressel & Misler, 1990): a rise in ambient glucose (to 10–15 mM) provoked, within 1–3 min, a train of extracellular Na^+-dependent action potentials which rapidly gave way to a plateau depolarization. (Rodent islets, despite expressing voltage-dependent Na^+ currents, display prolonged rhythmic bursting electrical activity consisting of Ca^{2+} action potentials which give rise to plateau depolarizations only at glucose concentrations greater than 20 mM.) In a previous publication (Pressel & Misler, 1991), we presented evidence which strongly suggested that this novel electrical ac-

tivity, just like the more classical electrical activity of rodent β-cells, is central to stimulus-secretion coupling. Electrical activity is initiated by the closure of ATP-sensitive K^+ channels and, subsequently, triggers Ca^{2+} entry which can support biphasic secretion. Here we present data suggesting that a prominent *permissive* effect of cAMP-enhancing agents on secretogogue-induced insulin secretion is exerted at the link between Ca^{2+} entry and insulin granule exocytosis, a step where these agents exert a substantial enhancing effect in islet species which secrete in response to fuel metabolites alone. Taken together, our observations suggest that the cellular cascade of stimulus-secretion coupling in canine β-cells is essentially similar to that in rodents. The seemingly unusual features of the cascade may help to more finely tune canine insulin secretion to its specific needs.

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We dedicate this paper, in loving memory, to Rose Misler.

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