Relative contribution of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms to the regulation of insulin secretion by glucose

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Abstract Although insulin secretion is usually regarded as a Ca^{2+}-dependent mechanism, recent studies have suggested the existence of a Ca^{2+}-independent pathway of regulation by glucose. Here, mouse islets were used to compare the contribution of Ca^{2+}-dependent and -independent pathways. Glucose increased insulin release in a concentration-dependent manner both in a control medium, when it depolarizes β cells and raises $[Ca^{2+}]_i$ (triggering signal), and in the presence of 30 mM K^+ and diazoxide, when it does not further raise $[Ca^{2+}]_i$ but increases its efficacy on exocytosis. Both Ca^{2+}-dependent responses were amplified by glucagon-like peptide-1+acetylcholine, and were strongly potentiated by forskolin+PMA. Under conditions of mild or stringent Ca^{2+} deprivation, glucose had no effect either alone or with GLP-1 and acetylcholine, and was poorly effective even during pharmacological activation of protein kinases A and C. Similar results were obtained with rat islets. It is concluded that physiological regulation of insulin release by glucose is essentially achieved through the two Ca^{2+}-dependent pathways without significant contribution of a Ca^{2+}-independent mechanism.

Key words: Insulin release; Stimulus-secretion coupling; Calcium; Glucose; Protein kinase; Pancreatic islet

1. Introduction

Pancreatic β cells are fuel-sensing cells under hormonal and neural control [1–5]. This explains the complexity of the mechanisms regulating insulin secretion. Besides classical transduction pathways involved in the response to hormones and neurotransmitters, sophisticated systems have evolved to couple fuel detection to the generation of secretory signals. The stimulation of insulin secretion by glucose requires metabolism of the sugar by the β cell and involves at least two mechanisms. The best characterized and major one serves to produce the triggering signal of exocytosis, a rise in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), through the following sequence of events [6–9]. Glucose metabolism generates signals, among which an increase in the ATP/ADP ratio [10], which close ATP-sensitive K^+ channels (K^+-ATP channels) in the plasma membrane. This leads to depolarization, opening of voltage-dependent Ca^{2+} channels and acceleration of Ca^{2+} influx. The second mechanism has been identified during treatment of β cells with diazoxide to hold K^+-ATP channels open, and high K^+ to depolarize the membrane and clamp $[Ca^{2+}]_i$ at an elevated, stable level [11,12]. Under these conditions glucose was found to increase the efficacy of Ca^{2+} on the secretory process [13]. The existence of this second mechanism is now widely accepted [14–16], but its mechanisms are still incompletely understood.

Studies using permeabilized cells [17,18] and membrane capacitance measurements [19,20] have confirmed that Ca^{2+} is a triggering signal of exocytosis, but have also shown that exocytosis can be induced in a Ca^{2+}-independent manner by poorly hydrolyzable GTPγS [21–23]. Recent studies have reported that glucose increases insulin release from intact rat islets and insulin-secreting HIT cells incubated or perifused under conditions of stringent Ca^{2+} deprivation. The necessary condition to disclose this effect was a combined activation of protein kinases A and C (PKA and PKC). These studies prompted the provocative suggestion that glucose also regulates insulin secretion by a third, Ca^{2+}-independent, mechanism [24,25].

It is essential to evaluate the potential importance of such a pathway of regulation of insulin secretion by glucose. We, therefore, compared the effects of various glucose concentrations on insulin release from mouse and rat islets incubated under selected conditions: in a control medium when the regulation is mainly dependent on the activity of K^+-ATP channels, in the presence of 30 mM K^+ and diazoxide to test the second mechanism of regulation, and under mild (diazoxide and nimodipine) and stringent (no Ca^{2+}+EGTA) conditions of Ca^{2+} deprivation. Glucose was tested alone, during physiological, receptor-mediated, activation of PKA and PKC with glucagon-like peptide-1 and acetylcholine [26–28], and during strong activation of the kinases with PMA (acting directly on PKC) and forskolin (stimulating adenylyl cyclase).

2. Materials and methods

2.1. Preparation and solutions

Islets were isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g) or fed female Wistar rats (200–250 g), followed by hand-picking.

Three types of solutions were used. For islet isolation and for control preincubations and incubations, the medium was a bicarbonate-buffered solution that contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 24 mM $NaHCO_3$. The high-K^+ medium contained 30 mM KCl and only 94.8 mM NaCl to maintain isoosmolarity. These solutions were supplemented with 250 μM diazoxide to ensure optimal control of the membrane potential and permit the K^+-ATP channel-independent effect of glucose to be studied [13]. Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$ and adding 1 mM EGTA. All solutions were gassed with O_2/CO_2 (94:6) to maintain pH 7.4, and contained bovine serum albumin (1 mg/ml).

After isolation the islets were subjected to two preincubations and a final incubation, all at 37°C. The first preincubation of 30 min was always carried out in control medium containing 15 mM glucose, and served to recover from the isolation procedure. The islets were then distributed in batches of three and transferred into glass tubes containing 1 ml medium for a second preincubation of 60 min, in control medium containing 3 mM glucose alone (A and B), in control medium containing 3 mM glucose, 5 μM nimodipine and 250 μM diazoxide

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(C), or in Ca^{2+}-free medium containing 3 mM glucose alone (D). At the end of this second preincubation, the medium was completely removed, discarded, and replaced by 1 ml of medium for a final incubation of 60 min. This medium, which contained various glucose concentrations and was supplemented with test substances (PMA, forskolin, acetylcholine, GLP-1), was a control medium (A), a high-K^+ medium (B), a control medium with nimodipine and diazoxide (C) or a Ca^{2+}-free medium (D). This procedure avoided manipulation of islets that become fragile after prolonged incubation in the absence of Ca^{2+}. At the end of the incubation, a portion of the medium was taken and appropriately diluted before insulin measurement by radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark). In one series of experiments, the final incubation medium was supplemented with sodium azide or adrenaline.

2.2. Reagents

Diazoxide was obtained from Schering-Plough Avondale (Rathdrum, Ireland); nimodipine from Bayer (Wuppertal, Germany); forskolin from Calbiochem (San Diego, CA); adrenaline from Parke-Davis (Zaventem, Belgium); sodium azide from Merck A.G. (Darmstadt, Germany); EGTA, phorbol 12-myristate 13-acetate (PMA), acetylcholine chloride (ACh) and 7–36 glucagon-like peptide-1 amide (hereafter abbreviated GLP-1) from Sigma Chemical Co. (St. Louis, MO).

2.3. Presentation of results

Results are presented as means ± S.E.M. for the indicated number of batches of islets. The statistical significance of differences between means was assessed by analysis of variance, followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Experiments with mouse islets

Under control conditions (normal Ca^{2+} and K^+), when the regulation of insulin secretion primarily depends on K^+-ATP channels, the stimulation by glucose displayed its characteristic sigmoidal dose-response relationship (Fig. 1A). When 25 nM GLP-1 and 1 μM ACh were added to the medium to activate PKA and PKC, basal release in the absence of glucose was increased 2.5-fold (0.65 ± 0.10 vs. 0.26 ± 0.03 ng/islet/h), the K_m of the response was decreased from 16 to ~11 mM, and the response to 30 mM glucose was doubled (9.6 ± 0.6 vs. 4.8 ± 0.4 ng/islet/h). The combination of 1 μM forskolin and 25 nM PMA similarly shifted the K_m, but increased basal and maximum responses 7-fold. The effects of a pharmacological activation of PKA and PKC are thus considerably larger than those of the physiological agents (Fig. 1A).

When the β cell membrane is depolarized with 30 mM K^+ in the presence of diazoxide, the K^+-ATP channel independent, Ca^{2+}-dependent effect of glucose can be studied [11–13]. Under these conditions, insulin release was markedly stimulated even in the absence of glucose (3.8 ± 0.3 ng/islet/h), but the sugar still caused a concentration-dependent increase in secretion (K_m ~13 mM) (Fig. 1B). GLP-1 and ACh had no effect on high K^+-induced insulin secretion in the low range of glucose concentrations, but produced a 30–40% potentiation ($P < 0.01$) in the presence of 15–30 mM glucose. The combination of forskolin and PMA was much more potent, increasing the response in the absence of glucose by 6.5-fold. However, a dose-dependent effect of glucose persisted under these conditions (Fig. 1B), which supports our previous conclusion that the second pathway of glucose regulation of insulin release is not mediated by an activation of PKA and PKC [13].

Blockade of Ca^{2+} influx through voltage-dependent Ca^{2+} channels by nimodipine [29] and diazoxide [11] abrogated glucose-induced insulin release (Fig. 1C). The low rate of secretion was increased by GLP-1 and ACh ($P < 0.05$), but the effect of glucose was limited to a doubling ($P < 0.05$) between 7 and 15 mM glucose. At 15 mM glucose, insulin secretion was 12-fold smaller than when Ca^{2+} entry was not inhibited (compare Figs. 1C and 1A). Again, the effect of forskolin and PMA was much larger than that of GLP-1 and ACh, and it was augmented by 3 and 7 mM glucose. In fact, the secretion rates were similar whether forskolin and PMA were tested in the absence or presence of diazoxide and nimodipine in the range 0–7 mM glucose, i.e. when Ca^{2+} influx was not stimu-

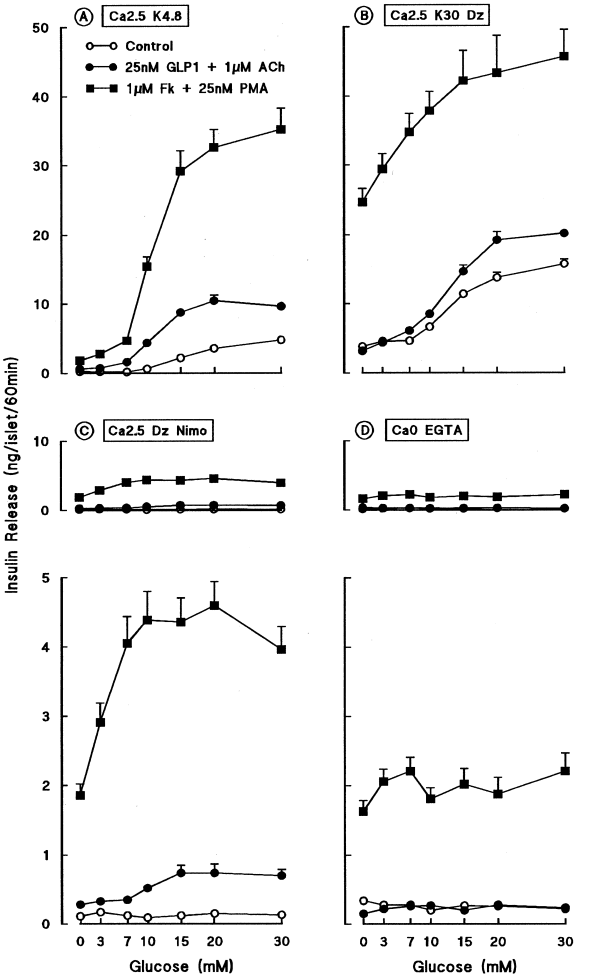

Fig. 1. Concentration dependence of the effect of glucose on insulin release by incubated mouse islets. Batches of three islets were preincubated for 60 min in a control medium containing 3 mM glucose alone (A and B) or supplemented with 5 μM nimodipine and 250 μM diazoxide (C), or in a Ca^{2+}-free medium containing 3 mM glucose and 1 mM EGTA (D). At the end of this preincubation, the medium was replaced by 1 ml of medium for a final incubation of 60 min. This medium, which contained various glucose concentrations was a control medium (A), a high-K^+ medium (B), a control medium with 5 μM nimodipine and 250 μM diazoxide (C), or a Ca^{2+}-free medium with 1 mM EGTA (D). As indicated, these media were also suplemented with 25 nM GLP-1 and 1 μM ACh (●), or 1 μM forskolin (Fk) and 25 nM PMA (■). After 60 min of incubation, an aliquot of the medium was taken for insulin assay. The upper and lower panels C and D show the same curves at the same scales as in A and B (upper panels) or at 10-fold larger scales (lower panels). Values are means ± S.E.M for 15–35 batches of islets from 3–7 separate experiments.

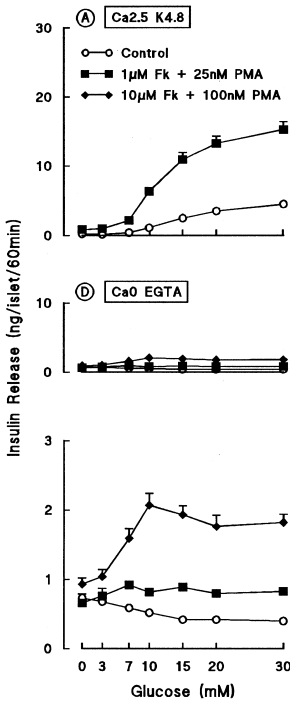

Fig. 2. Concentration dependence of the effect of glucose on insulin release by incubated rat islets. Batches of three islets were preincubated for 60 min in a control medium containing 3 mM glucose (A) or in a Ca^{2+}-free medium containing 3 mM glucose and 1 mM EGTA (D). At the end of this preincubation, the medium was replaced by 1 ml of medium for a final incubation of 60 min. This medium, which contained various glucose concentrations, was a control medium (A) or a Ca^{2+}-free medium (D). It was supplemented with forskolin (Fk) and PMA at the indicated concentrations. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. The two panels D show the same curves at the same scale as in A (upper panel) or at a 10-fold larger scale (lower panel). Values are means ± S.E.M for 15–30 batches of islets from 3–6 separate experiments.

lated. In contrast, higher glucose concentrations had an effect only when Ca^{2+} influx was not prevented (compare Figs. 1C and 1A). These characteristics are best explained by a sensitization of the releasing machinery to the action of Ca^{2+}.

Under conditions of stringent Ca^{2+} deprivation (no $CaCl_2$+EGTA), glucose did not affect insulin secretion either alone or in the presence of GLP-1 and ACh (Fig. 1D). In a glucose- and Ca^{2+}-free medium, forskolin and PMA approximately doubled secretion, as they did in the presence of Ca^{2+} without (Fig. 1A) or with diazoxide and nimodipine (Fig. 1C). In contrast, glucose was now without additional effect. At 15 mM glucose, insulin secretion was only 7% of that measured under control conditions (compare Figs. 1D and 1A).

To ascertain that insulin secretion observed in the absence of Ca^{2+} did not reflect unspecific leakage from damaged cells, we compared the inhibitory effects of energy deprivation and α_2-adrenoceptor activation under these four conditions. When azide, a mitochondrial poison, was used at the high concentration of 5 mM, insulin release was consistently blocked (Table 1). With 1 mM azide, however, the inhibition was strong when glucose was used alone, less in the presence of high K^+ and non-existent when forskolin and PMA were used in the absence of Ca^{2+} influx (diazoxide+nimodipine or Ca^{2+}-free medium). A low concentration of adrenaline (10 nM) inhibited insulin secretion by 20–40% under all conditions, and a high concentration (10 μM) consistently abolished insulin secretion (Table 1). These results show that regulated pathways underlie insulin release even when it is stimulated by forskolin and PMA under stringent Ca^{2+} deprivation [24]. It is also clear that physiological release induced by glucose in a control medium is the most sensitive to different physiological potentiators (GLP-1 and ACh) and inhibitors (adrenaline) as well as to energy deprivation.

3.2. Experiments with rat islets

As rat islets were used in the original study on which the description of the novel pathway for glucose regulation is based [24], a series of experiments was also performed with this model. Under control conditions, glucose increased insulin secretion in a concentration-dependent manner, to a maximum of about 20-fold. This stimulation was potentiated 4–5-fold by 1 μM forskolin and 25 nM PMA (Fig. 2A). In the absence of Ca^{2+}, insulin secretion was highest in the absence of glucose (0.73 ± 0.06 ng/islet/h) and decreased by about 40% when the concentration of the sugar was raised ($P < 0.05$ at 7 mM glucose). When the medium was supplemented with 1 μM forskolin and 25 nM PMA, the rate of secretion remained similar between 0 and 30 mM glucose. At 15 mM glucose, insulin secretion was only 8% of that measured under control conditions (compare Figs. 2D and 2A). When forskolin and PMA were used at higher concentrations of 10 μM and 100 nM respectively, insulin secretion was doubled between 0 and 10 mM glucose, and then plateaued (Fig. 2D).

Table 1
Effects of sodium azide and adrenaline on insulin release from mouse islets stimulated by 15 mM glucose under different conditions

Incubation conditions (mM)			Insulin release (ng/islet/60 min or percentage of controls)				
Ca^{2+}	K^+	Others	Controls	Sodium azide		Adrenaline	
				1 mM	5 mM	10 nM	10 μM
2.5	4.8	–	3.1 ± 0.2	0.8 ± 0.1 (26 ± 3%)	0.2 ± 0.1 (5 ± 1%)	1.6 ± 0.2 (57 ± 9%)	0.2 ± 0.1 (9 ± 2%)
2.5	30	Dz	8.8 ± 0.6	4.3 ± 0.3 (49 ± 3%)	0.3 ± 0.1 (3 ± 1%)	7.2 ± 0.5 (81 ± 5%)	0.9 ± 0.2 (9 ± 2%)
2.5	4.8	Dz+Nimo PMA+Fk	4.5 ± 0.4	4.2 ± 0.3[a] (98 ± 7%)	0.3 ± 0.1 (6 ± 1%)	3.6 ± 0.4 (82 ± 8%)	0.3 ± 0.1 (5 ± 2%)
0	4.8	PMA+Fk	1.8 ± 0.1	1.7 ± 0.1[a] (100 ± 8%)	0.3 ± 0.1 (17 ± 4%)	1.0 ± 0.1 (61 ± 7%)	0.4 ± 0.1 (22 ± 3%)

The concentration of glucose was 15 mM and test substances were present at the following concentrations: Dz (diazoxide) 250 μM, Nimo (nimodipine) 5 μM, PMA 25 nM, Fk (forskolin) 1 μM. EGTA (1 mM) was added to the medium without Ca^{2+}. Values are means ± S.E.M for 20 batches of islets (1 mM azide and 10 nM adrenaline) or 10 batches of islets (5 mM azide and 10 μM adrenaline). The percentage of change was calculated relative to controls within the same experiments. [a]Not significantly different from controls.

Very strong stimulation of PKA and PKC was thus necessary for glucose to increase insulin release in the absence of Ca^{2+}, and this small effect was restricted to low concentrations of the sugar as previously reported [24].

4. Discussion

The present study evaluated whether a Ca^{2+}-independent pathway [24,25] might significantly contribute to the regulation of insulin secretion by glucose. In rat islets, glucose produced an 18-fold increase in insulin secretion whether the control medium contained or not 1 μM forskolin and 25 nM PMA, but was without effect after Ca^{2+} deprivation. It was only when PKA and PKC were strongly stimulated in the presence of high concentrations of forskolin and PMA that a small effect of glucose on insulin release (doubling at 10 mM) was observed. In mouse islets, glucose was ineffective in a Ca^{2+}-free medium with EGTA. In the absence of glucose, pharmacological activation of PKA and PKC increased insulin release to a similar extent whenever Ca^{2+} influx was not stimulated. However, increasing glucose was without effect under stringent Ca^{2+} deprivation, similarly effective up to 7 mM in the absence or presence of diazoxide and nimodipine, and very effective up to 30 mM only when Ca^{2+} influx was not impeded. While strong activation of the kinases is thus able to increase insulin release in a glucose- and Ca^{2+}-independent manner, the effect of glucose can be ascribed to a potentiation of the action of basal and elevated $[Ca^{2+}]_i$.

Unphysiological experimental conditions are sometimes required for the identification of subtle regulatory pathways. It is then essential to establish whether these pathways also contribute to the physiological response. The second mechanism of action of glucose, the K^+-ATP channel-independent, Ca^{2+}-dependent mechanism, is most easily studied under conditions where $[Ca^{2+}]_i$ is clamped at an elevated level (11–13). However, the amplification of the action of Ca^{2+} by glucose can be observed over a range of $[Ca^{2+}]_i$ spanning that of the oscillations occurring in β cells stimulated by glucose in a control medium [30]. We suggest that this pathway serves to optimize the efficacy of the increase in $[Ca^{2+}]_i$. On the other hand, we cannot evaluate what proportion of the secretory response to a given glucose concentration is attributable to the rise in $[Ca^{2+}]_i$ and to the change in Ca^{2+} efficacy.

If a Ca^{2+}-independent mechanism of regulation [24,25] is operative only in the presence of extremely low $[Ca^{2+}]_i$, much lower than the concentrations ever occurring in β cells in vivo, it is an interesting curiosity without physiological relevance. If this mechanism is also operative in the presence of physiological $[Ca^{2+}]_i$, its contribution could be hidden in the overall secretory response. Then several questions arise. First, is the mechanism really Ca^{2+}-independent or is it similar to the glucose-induced amplification of the action of Ca^{2+} (second mechanism)? This is unlikely because this Ca^{2+}-dependent effect of glucose does not require activation of PKA and PKC [13] and persists under strong activation of these kinases (see Fig. 1B). Second, if the mechanism is distinct from the others, what is its quantitative importance as compared with the Ca^{2+}-dependent mechanisms? This contribution is likely to be minimal because, even under optimal conditions, the amount of insulin that can be released in the absence of Ca^{2+} does not exceed a small percentage of the appropriate controls. In recent experiments using capacitance measurements to monitor exocytosis of insulin granules, the Ca^{2+}-independent release induced by GTPγS was also much smaller than that induced by Ca^{2+} [23]. Third, could a physiological activation of both PKA and PKC ever be sufficient (equivalent to that produced by pharmacological agents) for the Ca^{2+}-independent effect to occur in vivo? This is most unlikely, whereas a much milder, separate activation of each kinase is sufficient to potentiate markedly Ca^{2+}-dependent exocytosis. This has been shown by direct measurements of insulin release from intact [31–33] or permeabilized cells [34,35] and by capacitance measurements [19,36]. In experiments using the latter approach, forskolin and PMA individually amplified the exocytotic response to Ca^{2+} influx whereas their combination had no effect in the absence of depolarization-induced Ca^{2+} rise [36].

In conclusion, although the complex sequence of events leading to insulin secretion may include Ca^{2+}-independent steps, the physiological regulation by glucose is essentially achieved through the two Ca^{2+}-dependent pathways (rise in β cell $[Ca^{2+}]_i$ and increase in Ca^{2+} efficacy), with little, if any, contribution of a Ca^{2+}-independent mechanism.

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