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EXTRACELLULAR Ca^{2+} INDUCES A RAPID INCREASE IN CYTOPLASMIC FREE Ca^{2+} IN PANCREATIC β-CELLS

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Using pancreatic β-cells isolated from obese hyperglycemic mice, it was demonstrated that the addition of 5 mM extracellular Ca^{2+} evoked a rapid and transient increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). The effect remained in the presence of D-600. Extracellular Ca^{2+} did not raise $[Ca^{2+}]_i$ subsequent to emptying the inositol 1,4,5-trisphosphate ($InsP_3$) sensitive pool by carbamylcholine stimulation, indicating that the pool released by extracellular Ca^{2+} is of similar origin. Stimulation with extracellular Ca^{2+} was accompanied by a pronounced insulin release. Our results suggest that the Ca^{2+}-induced rise in $[Ca^{2+}]_i$ is mediated through the formation of $InsP_3$, a mechanism that might operate also in other types of cells.

It is well established that stimulation of insulin secretion in response to most secretagogues is critically dependent on the presence of extracellular Ca^{2+} (1). Indeed, supraphysiological concentrations of Ca^{2+} promotes insulin release in a variety of experimental situations (1-3). Glucose is believed to stimulate insulin release by inactivating ATP-regulated K^+ channels, resulting in a depolarization-induced opening of voltage-activated Ca^{2+} channels and consequently an increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (4-8). Recently, it was demonstrated that high extracellular concentrations of Ca^{2+} transiently reversed the reduction in $[Ca^{2+}]_i$ evoked by the α_2-adrenergic agonist clonidine (9,10). Interestingly, this effect was only partially inhibited by D-600 (11), a blocker of voltage-activated Ca^{2+} channels, suggesting that extracellular Ca^{2+} may raise $[Ca^{2+}]_i$ by other means than influx through voltage-activated Ca^{2+} channels. In the present study we were interested in establishing this phenomenon, and getting a better understanding of the underlying mechanism(s). We therefore decided to investigate the effects of high concentrations of extracellular Ca^{2+} on $[Ca^{2+}]_i$, membrane potential and insulin release in pancreatic β-cells isolated from obese hyperglycemic mice.

MATERIALS & METHODS

Animals and preparation of cells: Adult obese hyperglycemic mice (ob/ob) of both sexes were taken from a local non-inbred colony (12) and starved overnight.

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The islets were isolated with collagenase and a cell suspension was prepared and cultured overnight as previously described (13).

Media: The medium used was a Hepes buffer, pH 7.4, containing 1.28 mM Ca^{2+} and with Cl^- as the sole anion (14). In all experiments except for measurements of membrane potential, the medium was supplemented with 1 mg/ml bovine serum albumin.

Measurements of $[Ca^{2+}]_i$ and membrane potential: Cell suspensions were incubated for 45 min with 5 μM quin 2/acetoxymethylester (Sigma Chemical Co., St. Louis, MO., U.S.A.). This resulted in a loading of approximately 1.3 nmol quin 2 per 10^6 cells (11). The suspensions were washed twice before being resuspended in 1.3 ml medium. The excitation and emission wavelengths were 340 and 490 nm, respectively and calibration was performed as previously described (15). Qualitative changes in membrane potential were estimated with bisoxonol (Molecular Probes, Junction City, OR., U.S.A.) (16), used at a concentration of 150 nM. Cell suspensions were washed twice before being resuspended in 1 ml medium and fluorescence was recorded at excitation and emission wavelenghts of 540 and 580 nm, respectively. All measurements were made at 37°C in 1 cm polystyrene cuvettes in an Aminco-Bowman spectrofluorometer, slightly modified to allow constant stirring. All traces shown are typical for experiments repeated with at least three different cell preparations.

Measurements of insulin release: The dynamics of insulin release were studied by perifusing 0.5-1 x 10^6 cells mixed with Bio-Gel P4 polyacrylamide beads in a 0.5 ml column at 37°C (17). The flow rate was 0.3 ml/min and 1-2 min fractions were collected and analysed for insulin radioimmunologically, using crystalline rat insulin as the standard. The data shown are typical for experiments performed with three different cell preparations.

RESULTS

Figure 1A shows that the increase in $[Ca^{2+}]_i$ induced by 20 mM glucose was reversed by 400 μM diazoxide, a sulfonamide drug that inhibits insulin release by activating the ATP-regulated K^+ channels (18). Such an activation leads to repolarization with subsequent closure of the voltage-activated Ca^{2+} channels and a reduction in $[Ca^{2+}]_i$ (8,19). Addition of 5 mM Ca^{2+} extracellularly produced a rapid and transient increase in $[Ca^{2+}]_i$ which gradually declined to a new level, yet still higher than before stimulation. The addition of 100 μM carbamylcholine, a muscarinic receptor agonist, resulted in a similar transient Ca^{2+} peak, after which $[Ca^{2+}]_i$, also in this case, remained slightly elevated. Under these conditions, neither Ca^{2+} nor carbamylcholine stimulation affected membrane potential (Fig. 1C). As is shown in figure 1B the addition of carbamylcholine, in the presence of glucose and diazoxide, elicited a pronounced and fast rise in $[Ca^{2+}]_i$, but in this case subsequent addition of 5 mM Ca^{2+} induced only a slow and small increase. Again, no effect was observed on membrane potential (Fig. 1D). In experiments not illustrated here, atropine did not affect the response to Ca^{2+} whereas the effect of carbamylcholine was completely blocked.

In figure 2, the glucose induced rise in $[Ca^{2+}]_i$ was reversed by directly blocking the voltage-activated Ca^{2+} channels with 50 μM D-600. In this situation as well, $[Ca^{2+}]_i$ was increased by extracellular Ca^{2+} and carbamylcholine (Fig. 2A). Also

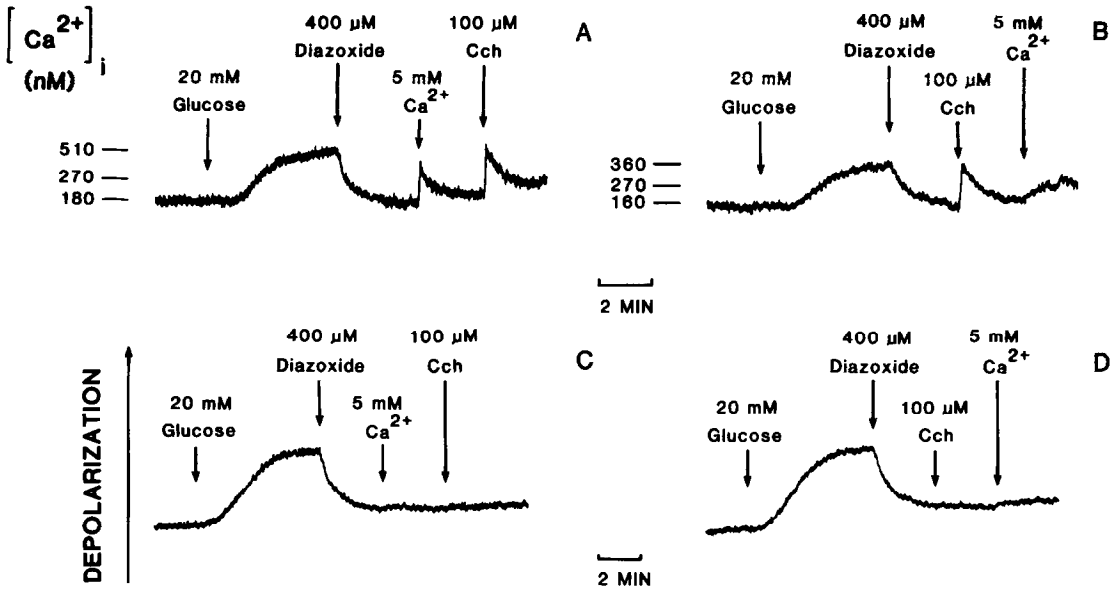

Fig. 1. Effects of glucose, diazoxide, Ca^{2+} and carbamylcholine (Cch) on $[Ca^{2+}]_i$ (traces A and B) and membrane potential (traces C and D). Note the different time scales for the measurements of $[Ca^{2+}]_i$ and membrane potential, respectively.

in the presence of D-600 a previous exposure to carbamylcholine prevented the transient increase in $[Ca^{2+}]_i$ induced by high Ca^{2+} (Fig. 2B).

Since certain divalent cations have been shown to share common properties with Ca^{2+} in the β-cell (20-22), the effects of high concentrations of Ba^{2+}, Sr^{2+} and Mg^{2+} on $[Ca^{2+}]_i$, were investigated in the presence of glucose and D-600.

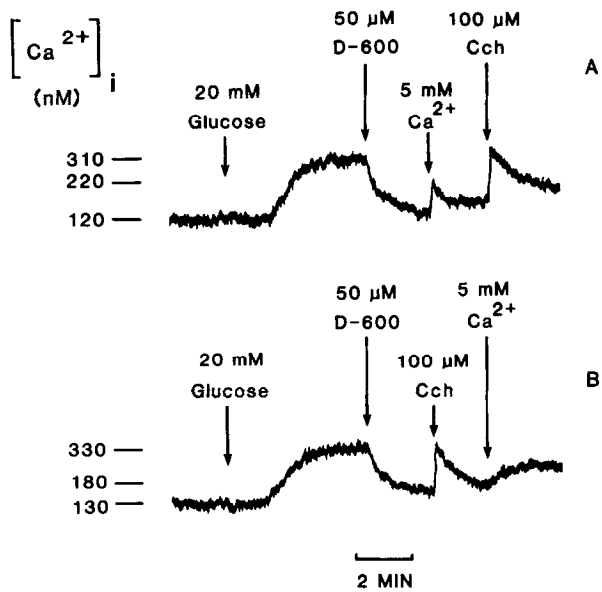

Fig. 2. Effects of glucose, D-600, Ca^{2+} and carbamylcholine (Cch) on $[Ca^{2+}]_i$.

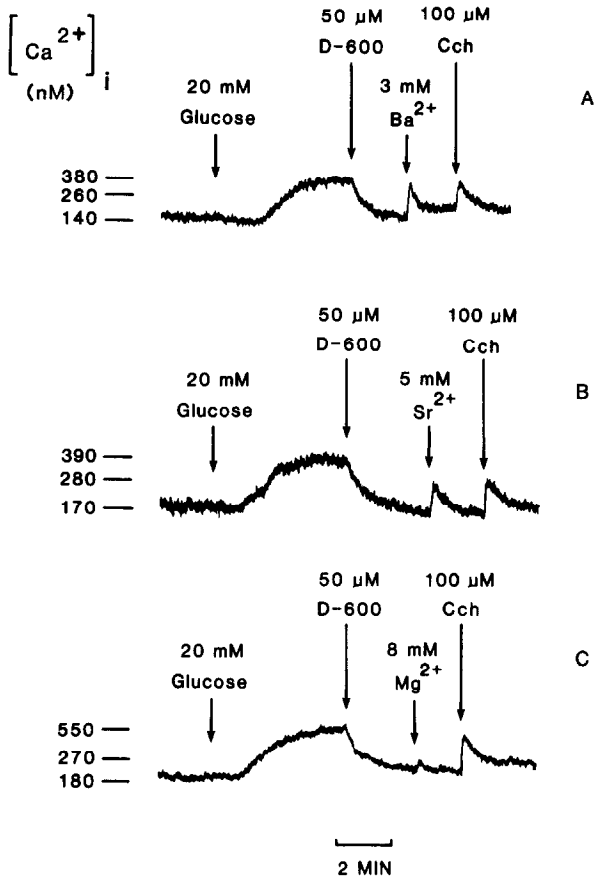

Fig. 3. Effects of glucose, D-600, Ba^{2+}, Sr^{2+}, Mg^{2+} and carbamylcholine (Cch) on $[Ca^{2+}]_i$.

As is shown in figure 3A, Ba^{2+} was effective in eliciting a Ca^{2+} peak at a concentration of 3 mM. To induce a similar response with Sr^{2+} 5 mM was required (Fig. 3B), whereas 8 mM Mg^{2+} hardly affected $[Ca^{2+}]_i$ at all (Fig. 3C). When carbamylcholine was added prior to Ba^{2+} or Sr^{2+}, these ions failed to increase $[Ca^{2+}]_i$ (data not shown).

When studying the dynamics of insulin release in a perifusion system, the stimulatory action of 20 mM glucose was rapidly abolished by D-600 (Fig. 4). In panel A it is shown that addition of 5 mM Ca^{2+} induced insulin release which remained stimulated for a longer period of time than $[Ca^{2+}]_i$ was elevated (cf Fig. 2A). When 7.5 mM NaCl was used as an osmotic control for Ca^{2+}, secretion was not stimulated (Fig. 4B). As can be noted in figure 4C, carbamylcholine elicited a large and rapidly declining peak of insulin release.

DISCUSSION

The present investigation demonstrates for the first time, a Ca^{2+}-induced rise in $[Ca^{2+}]_i$ in pancreatic β-cells. This effect showed no strict dependency on

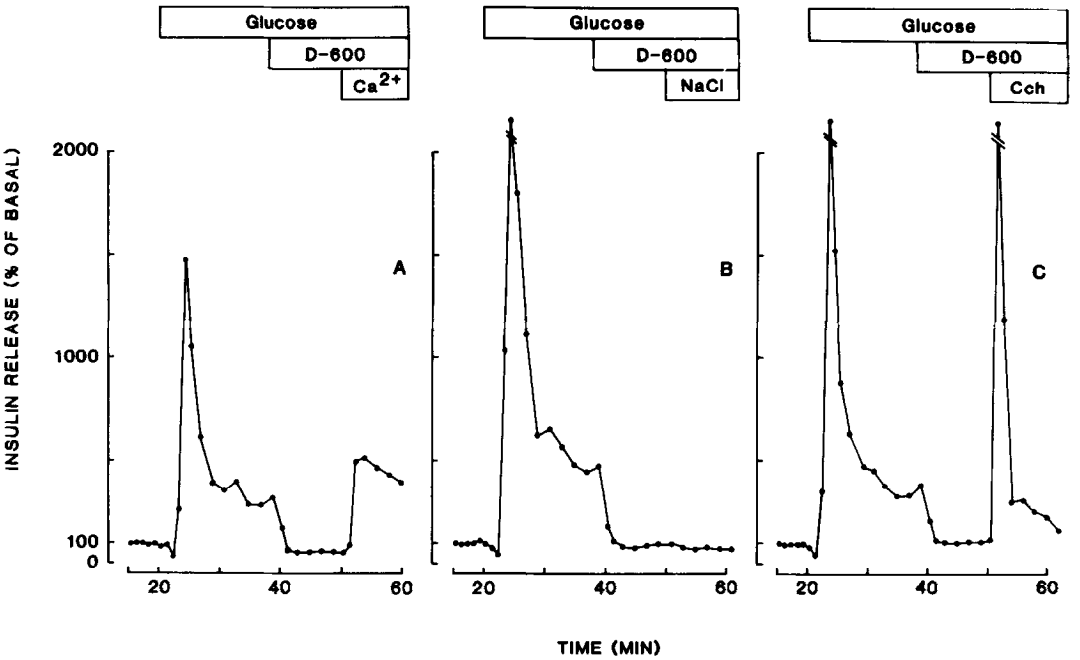

Fig. 4. Effects of 20 mM glucose, 50 μM D-600, 5 mM Ca^{2+}, 7.5 mM NaCl and 100 μM carbamylcholine (Cch) on the kinetics of insulin release from perifused β-cell aggregates. The results are presented as percentage of the average insulin release during the 5 min period preceding the introduction of glucose.

membrane depolarization, since similar results were obtained either in the presence of diazoxide or D-600. Neither did high concentrations of Ca^{2+} per se increase membrane potential. Since the transient increase in $[Ca^{2+}]_i$, in response to extracellular Ca^{2+}, was not blocked by D-600, it is not explained by Ca^{2+} influx through voltage-activated Ca^{2+} channels. Although the late increase in $[Ca^{2+}]_i$ subsequent to stimulation with extracellular Ca^{2+} probably reflects unspecific leakage of Ca^{2+} into the cells, this is unlikely to account for the $[Ca^{2+}]_i$ peak, in view of both transiency and rapidity in onset.

An attractive explanation for the transient increase in $[Ca^{2+}]_i$ is that high extracellular concentrations of Ca^{2+} promotes mobilization of internally stored Ca^{2+}. The best way to demonstrate such an effect, is to perform the experiments in the absence of extracellular Ca^{2+}. For obvious reasons this strategy would be futile using Ca^{2+} as a stimulator. To our discontent, it was neither possible to draw any final conclusions about the origin of the rise in $[Ca^{2+}]_i$ when stimulating the cells with the Ca^{2+} analogues Ba^{2+} and Sr^{2+} under these conditions. This was mainly due to the fact that the latter ions increased quin 2 fluorescence in a Ca^{2+} deficient medium, both by saturating extracellular indicator and by entering the cells, to the extent that possible fluorescence changes reflecting true changes in $[Ca^{2+}]_i$ were effectively masked. It is of interest to note that previous studies

have indeed demonstrated that 2.5 mM Ba^{2+} stimulated ^{45}Ca efflux from preloaded pancreatic islets perifused with a Ca^{2+} deficient medium (20).

It has been clarified that carbamylcholine stimulation in pancreatic β-cells leads to release of Ca^{2+} from internal stores, an effect mediated through the formation of inositol 1,4,5-trisphosphate ($InsP_3$) (23-27). The dynamics of the $[Ca^{2+}]_i$ increase in response to Ca^{2+}, or its analogues Ba^{2+} and Sr^{2+}, were similar to those obtained by carbamylcholine stimulation and it is tempting to speculate that this increase is as well mediated by $InsP_3$. Accordingly, neither Ca^{2+}, Ba^{2+} nor Sr^{2+} were able to induce a rise in $[Ca^{2+}]_i$ subsequent to stimulation with a high concentration of carbamylcholine, probably due to depletion of the Ca^{2+} pool sensitive to $InsP_3$. In this context it should be noted that carbamylcholine still produced a rise in $[Ca^{2+}]_i$ subsequent to stimulation with the divalent cations. This should not be taken as an argument against the notion that Ca^{2+}-induced Ca^{2+} release is mediated through generation of $InsP_3$, but may simply reflect a difference in the ability to promote $InsP_3$ formation. The suggestion that the actual concentration of carbamylcholine is a more potent stimulator of $InsP_3$ formation is also supported from the results always demonstrating a more pronounced Ca^{2+} response by the agonist, compared to the effects evoked by extracellular Ca^{2+}. It can be assumed that the initial pronounced peak of insulin release, in response to carbamylcholine stimulation, results from an $InsP_3$ evoked increase in $[Ca^{2+}]_i$. The corresponding release in response to extracellular Ca^{2+} was much smaller, which is also to be expected if Ca^{2+} stimulation is accompanied with only a restricted formation of $InsP_3$. Moreover, insulin release evoked by extracellular Ca^{2+} remained stimulated for a longer period of time than the peak increase in $[Ca^{2+}]_i$, indicating that $[Ca^{2+}]_i$ is not always a moment-to-moment regulator of insulin secretion (28,29).

The transient effects of Ca^{2+}, Ba^{2+} and Sr^{2+} on $[Ca^{2+}]_i$ in the β-cells resemble those previously reported for fura 2 loaded parathyroid cells (30). In these cells it is believed that the divalent cations excert their effects by binding to a cell surface Ca^{2+} receptor (30). The extracellular concentration of Ca^{2+} is the primary physiological stimulus regulating secretion of parathyroid hormone and the existence of such receptors would enable the parathyroid cell to detect fluctuations in plasma Ca^{2+} levels. Although exciting, the significance of the Ca^{2+}-induced Ca^{2+} release in the physiological regulation of insulin release is not easily envisaged. Whether the divalent cation-induced rise in $[Ca^{2+}]_i$ in the β-cells, is mediated through an interaction with a putative Ca^{2+} receptor is at the moment not known. It should be remembered that previous studies on pancreatic islets have demonstrated that Ca^{2+} influx subsequent to K^+ depolarization promotes the formation of $InsP_3$ (25). The extent to which this type of Ca^{2+}-induced Ca^{2+} release also exists in other cell types, can so far just be a matter of speculation.

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