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Background Ca^{2+} Influx Mediated by a Dihydropyridine- and Voltage-insensitive Channel in Pancreatic β-Cells

MODULATION BY Ni^{2+}, DIPHENYLAMINE-2-CARBOXYLATE, AND GLUCOSE METABOLISM*

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A stepwise increase in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) can evoke insulin release from pancreatic islets in the absence of secretagogues. We have investigated the ionic mechanism underlying this secretory response by recording intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) from single mouse islets of Langerhans using ratiometric fura-2 microfluorometry. In the presence of 11 mM glucose, the $[Ca^{2+}]_i$ undergoes fast oscillations associated with bursting electrical activity. Nifedipine (10 μM) suppressed these oscillations and markedly lowered the $[Ca^{2+}]_i$. Raising the $[Ca^{2+}]_o$ from 2.56 to 12.8 mM in the continued presence of 11 mM glucose and nifedipine evoked pronounced $[Ca^{2+}]_i$ rises of variable amplitude and time course. This effect was dose-dependent (EC_{50} = 3.6 mM) and remained essentially unchanged in the absence of glucose or in the presence of 3 mM glucose and nifedipine, conditions where β-cells are hyperpolarized by approximately −25 mV. Depleting the acetylcholine-mobilizable internal Ca^{2+} pools by repetitively challenging the islets with acetylcholine in the absence of Ca^{2+} actually potentiated the standard high Ca^{2+} responses. The latter were strongly reduced by millimolar concentrations of Ni^{2+} (70% reduction at 3 mM) and by diphenylamine-2-carboxylate (DPC; IC_{50} = 145 μM), a blocker of nonselective cation channels. The standard high Ca^{2+} responses were relatively insensitive to the glycolytic inhibitor mannoheptulose. It is proposed that the high Ca^{2+}-evoked $[Ca^{2+}]_i$ responses are primarily accounted for by Ca^{2+} influx through dihydropyridine- and voltage-insensitive, nonselective cation channels. These channels do not appear to be under the control of glucose metabolism. Although their function is unknown, they may be essential to supplying the β-cells with Ca^{2+} in the absence of stimulatory levels of fuel secretagogues.

Glucose-induced insulin release from pancreatic β-cells is totally dependent on the influx of Ca^{2+} from the extracellular medium (1, 2). Voltage-sensitive Ca^{2+} channels are well known to play a pivotal role in glucose-stimulated Ca^{2+} influx, with the dihydropyridine-sensitive (L-type) channel being the prevalent, if not exclusive, subtype at least in adult mouse pancreatic β-cells (3–5).

Raising the $[Ca^{2+}]_o$[1] above physiological levels has negligible effects on $[Ca^{2+}]_i$ in most cell types, suggesting that either resting Ca^{2+} permeability is low or that the $[Ca^{2+}]_i$ is under a particularly tight control by buffering/extrusion mechanisms, or both. In contrast, cell types specialized in sensing $[Ca^{2+}]_o$ changes (*e.g.* parathyroid cells and osteoclasts) appear to transduce these changes into prominent $[Ca^{2+}]_i$ rises through a plasma membrane "Ca^{2+} receptor" apparently coupled to Ca^{2+} release from intracellular stores and Ca^{2+} influx (6–11). Pancreatic β-cells isolated from obese hyperglycemic mice have been reported to display similar $[Ca^{2+}]_i$ rises when challenged with high Ca^{2+} in the presence of the voltage-sensitive Ca^{2+} channel blocker D-600 (12). These signals were strongly impaired by carbachol, leading to the hypothesis that Ca^{2+} release from internal stores might be involved (12).

Exposure to solutions containing high extracellular Ca^{2+} concentrations has been reported to evoke insulin release from perfused pancreases, isolated islets, and isolated β-cells in the absence of other secretagogues (2, 12–16; reviewed in Refs. 1 and 2). Raising external Ca^{2+} concentration is also known to stimulate $^{45}Ca^{2+}$ efflux from preloaded unstimulated islets (2, 16–18), a process that appears to be the consequence of enhanced Ca^{2+} influx through the plasma membrane and of a concomitant accumulation of the cation in the β-cell cytoplasm (2, 15). We have recently observed that exposure to high Ca^{2+} solutions depolarizes the β-cell membrane by approximately 10 mV in the presence of the dihydropyridine nifedipine (5, 19). These observations suggest that the resting β-cell membrane has a large basal permeability for Ca^{2+} and prompted a search for the possible involvement of Ca^{2+} influx mechanisms in the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise.

In the present study, we show that single pancreatic islets of Langerhans respond to high Ca^{2+} with pronounced $[Ca^{2+}]_i$ rises. These responses are relatively insensitive to glucose metabolism, are not dependent on Ins 1,4,5-P_3-linked internal Ca^{2+} stores and can be suppressed by Ni^{2+} and diphenylamine-2-carboxylate, both blockers of nonselective cation channels. We postulate that the DPC-sensitive channels serve the main function of providing pancreatic β-cells and other secretory cells with Ca^{2+}, especially in the absence of depolarizing secretagogues when voltage-sensitive Ca^{2+} channels are rendered essentially inoperative by membrane hyperpolarization.

EXPERIMENTAL PROCEDURES

Islet Isolation and Culture—Detailed accounts of the basic islet isolation procedures have been published (20). Briefly, 3- to 6-month-old female albino mice weighing 28–40 g (Charles River Breeding Labora-

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[1] The abbreviations used are: $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DPC, diphenylamine-2-carboxylate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; fura-2/AM, acetoxymethyl ester of fura-2.

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tories) were killed by a blow to the head, followed by cervical dislodgement. After exposing the abdominal cavity, the distal outlet of the common bile duct was collapsed. The duct was then cannulated close to the junction with the cholecystic and hepatic ducts, and the pancreas was distended by injecting approximately 4 ml of an ice-cold salt solution supplemented with approximately 1.4 mg/ml collagenase (type P, Boehringer Mannheim) and 3% bovine serum albumin (fraction V, protease-free, Sigma). This solution had the following composition: 115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.56 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25 mM HEPES, and 11 mM glucose (pH 7.4 after saturation with 5% CO_2/95% O_2). The injected pancreas was then dissected, transferred into a 15-ml plastic tube, and incubated at 37 °C under gentle manual shaking for 5–7 min. Collagenase digestion was stopped by adding a large excess of ice-cold solution supplemented with 3% bovine serum albumin. The islets and pieces of exocrine tissue were allowed to sediment, after which the supernatant was removed and a large excess of the solution added again. This washing step was repeated twice to free the solution from collagenase. Intact islets were then handpicked at room temperature using the tip of an automatic pipette, a process that was repeated twice to separate the islets from any contaminating pieces of exocrine tissue. The solutions used throughout the isolation procedure were routinely kept saturated in 95% O_2/5% CO_2.

For culture, the islets were handpicked and transferred into sterile RPMI-based medium, a process that was repeated three times to minimize the possibility of contamination. The culture medium had the following composition: RPMI 1640 containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 25 mM $NaHCO_3$, and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin), pH adjusted to 7.2 with NaOH to give 7.4 in the incubator (all products from Biological Industries, Beth Haemek, Israel). Typically, the islets were cultured in small Petri dishes containing 2 ml of medium (approximately 4–8 islets per dish). The islets were incubated overnight at 37 °C under a 5% CO_2/95% air-humidified atmosphere and were usually used in the experiments within 24 h from isolation.

Solutions—The standard salt solution used in the microfluorescence experiments had the following composition: 125 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 2.56 mM $CaCl_2$, and 1.1 mM $MgCl_2$, constantly gassed with 95% O_2/5% CO_2 for a final pH of 7.4. This solution was supplemented with different concentrations of glucose as required. In many instances, the external Ca^{2+} concentration was adjusted to different levels by adding, immediately before the experiments and under intense CO_2 bubbling, the required amounts of $CaCl_2$ to Ca^{2+}-free solutions. The solutions used to calibrate fura-2 fluorescence in terms of $[Ca^{2+}]_i$ were as previously described (5, 21).

Fura-2 Loading and Microfluorometry—Groups of 4–8 islets were typically incubated in the original culture medium supplemented with 4 μM fura-2/AM for 45 min at 37 °C (incubator), after which they were either transferred to a fast perfusion chamber (21) placed on the stage of an inverted epifluorescence microscope (Nikon Diaphot, Japan) or kept in fresh culture medium in the incubator for later use in the experiments (usually a significant fraction of the original fura-2 fluorescence was retained under these conditions, provided that the post-loading period did not exceed 90 min). The bottom of the chamber was previously coated with poly-L-lysine to facilitate islet attachment, which was usually achieved in less than 10 min under stationary conditions. The temperature in the chamber was 37 °C. The perifusion system used in the experiments and the respective temperature control stages have been described previously in detail (21). We found that protecting the islets from ambient light (including that from the microscope tungsten bulb) significantly improved the quality of the $[Ca^{2+}]_i$ responses to secretagogues.

The $[Ca^{2+}]_i$ was recorded from single islets using a dual-excitation microfluorescence system (Deltascan, PTI, Princeton, NJ) essentially as described previously for islets and single chromaffin cells (5, 21, 22). Briefly, fura-2 incorporated in the islet cells was excited at 340 and 380 nm via two monochromators. The fluorescence was detected by a photomultiplier after passing through a band-pass interference filter centered at 510 nm. The data were automatically corrected for background fluorescence and acquired at 10 Hz by a 386SX/16 MHz computer. The fluorescence ratio F_{340}/F_{380} was converted into $[Ca^{2+}]_i$ values by an *in vitro* calibration procedure essentially as described previously (5, 21, 22).

Materials—Fura-2/AM and fura-2 (free acid) were from Molecular Probes. An initial sample of DPC was gently provided by Dr. P. Poronnik (Dept. of Physiology, University of Sidney, Australia) and was later purchased from Aldrich-Chemie (Steinheim, Germany). Importantly, DPC was stored as a 0.1 M stock solution in dimethyl sulfoxide. Previous attempts to use DPC from a 0.5 M stock solution in dimethyl sulfoxide led to its precipitation in the form of small particles, presumably because the dimethyl sulfoxide/H_2O ratio in the final solution was insufficient to provide a stable solution. All other chemicals were from Sigma.

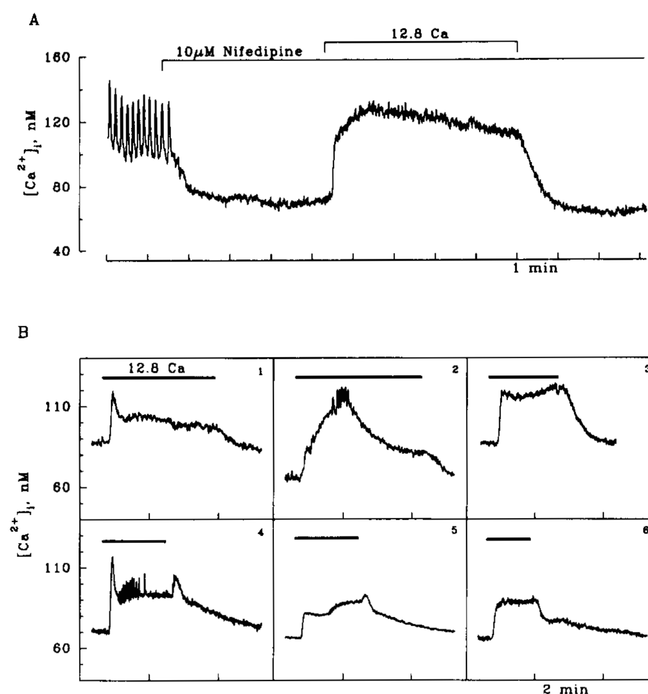

FIG. 1. **Effect of a stepwise increase in external Ca^{2+} concentration on the $[Ca^{2+}]_i$ in the presence of 11 mM glucose and nifedipine.** *Panel A* illustrates the basic protocol used in the experiments shown in *panel B*. *A*, the $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM in the continued presence of 11 mM glucose and nifedipine as indicated. The $[Ca^{2+}]_i$ was recorded from single islets of Langerhans using ratiometric fura-2 microfluorometry. *B*, the $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM (*islets 1–3*) or from 0.5 to 12.8 mM (*islets 4–6*) in the continued presence of 11 mM glucose and 10 μM nifedipine, as indicated by the *bars*. Preincubation time with nifedipine was 10 min in all cases.

RESULTS

We have investigated the effects of raising extracellular Ca^{2+} concentration on $[Ca^{2+}]_i$ in the presence of the L-type voltage-sensitive Ca^{2+} channel antagonist nifedipine. In these experiments, the $[Ca^{2+}]_i$ was recorded from fura-2-loaded whole islets of Langerhans.

In the presence of 11 mM glucose, the $[Ca^{2+}]_i$ undergoes fast oscillations (Fig. 1*A*), as previously reported (5, 22–24). These oscillations arise from Ca^{2+} influx taking place through voltage-sensitive Ca^{2+} channels during the depolarized phases of bursting electrical activity (24). Addition of nifedipine in the continued presence of 11 mM glucose rapidly suppressed the $[Ca^{2+}]_i$ oscillations and lowered the $[Ca^{2+}]_i$ by approximately 30 nM (Fig. 1*A*; average effect was 39 ± 20 nM (± S.D.), n = 18 different islets). This inhibitory effect is reminiscent of the spike-suppressing action of the dihydropyridine (5, 19) and is consistent with the reported prevalence of the L-type voltage-sensitive Ca^{2+} channel in mouse β-cells (3, 4).

Raising the $[Ca^{2+}]_o$ from 2.56 to 12.8 mM in the continued presence of 11 mM glucose and nifedipine evoked a pronounced $[Ca^{2+}]_i$ rise to a level close to the peak of the oscillations recorded in the absence of the dihydropyridine (Fig. 1*A*). In the example shown in Fig. 1*A*, the $[Ca^{2+}]_i$ exhibited a slight tendency to decay during persistent exposure to high Ca^{2+}. Fig. 1*B* shows that both the amplitude and the time course of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ transients are highly variable from islet to islet. Both experiments in which the basal $[Ca^{2+}]_o$ was 2.56 mM (islets 1 through 3) and 0.5 mM (islets 4 through 6) are shown in Fig. 1*B*. The predominant feature of the high Ca^{2+} response

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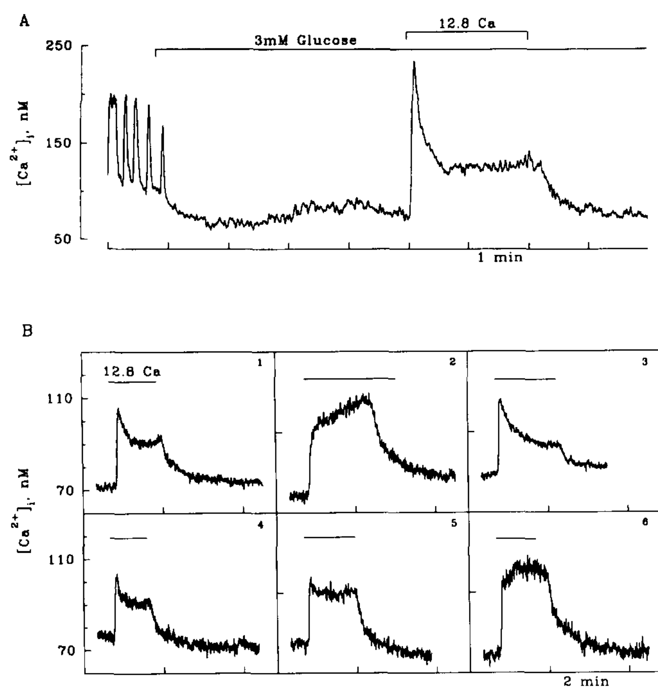

FIG. 2. **Effect of a stepwise increase in external Ca^{2+} concentration on the $[Ca^{2+}]_i$ at a substimulatory glucose level.** *Panel A* illustrates the basic protocol used in the experiments shown in *panel B*. *A*, the glucose concentration in the perifusate was reduced from 11 mM (oscillations) to 3 mM and was kept constant at the latter level throughout the experiment. The $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM in the continued presence of 3 mM glucose as indicated. *B*, the $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM in the absence (*islets 1–3*) and presence (*islets 4–6*) of 10 μM nifedipine (preincubation time with nifedipine was 10 min in all cases), as indicated by the *bars*. Glucose concentration was 3 mM throughout (*1–6*).

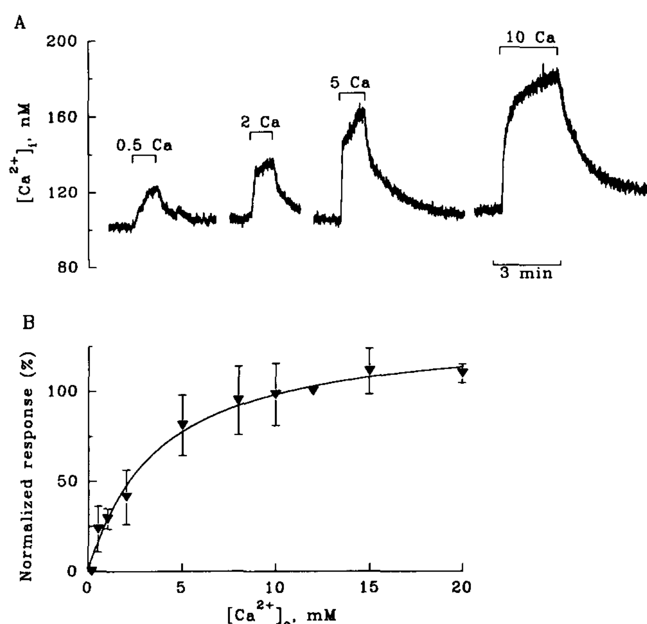

FIG. 3. **Dose dependence of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise.** *A*, the $[Ca^{2+}]_o$ was pulsed from 0.2 mM to the various concentrations indicated (0.5, 2, 5, and 10 mM), as depicted by the *brackets*. The solution contained 11 mM glucose and 10 μM nifedipine throughout. *B*, data pooled from experiments similar to that shown in the *upper panel*. The extent of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise was assessed by taking the difference between the level measured 60 s after the beginning of each pulse and the basal $[Ca^{2+}]_i$. This response was normalized within each experiment to that obtained with a 12 mM Ca^{2+} pulse (% normalized response) and was plotted against the size of the high Ca^{2+} pulses. The *vertical bars* represent ± S.D. (n = 3–4 different islets). Absolute response measured for a 12 mM Ca^{2+} pulse: 53 ± 11 nM (n = 4 islets). Also shown in the figure is the best fit to a saturation function.

was a prominent and rapid $[Ca^{2+}]_i$ peak, which was either followed by a decrease toward a plateau (*e.g.* islets 1 and 4 in Fig. 1*B*) or by a slow secondary $[Ca^{2+}]_i$ rise (*e.g.* islets 3 and 5). However, a fraction of the islets examined (5 out of 30) displayed relatively slow $[Ca^{2+}]_i$ rises to a plateau (*e.g. islet 2* in Fig. 1*B*). On average, the difference between the $[Ca^{2+}]_i$ recorded 60 s after the moment of exposure to high Ca^{2+} and the basal $[Ca^{2+}]_i$ amounted to 56 ± 35 nM and 67 ± 23 nM (n = 11 different islets) for pre-pulse Ca^{2+} levels of 2.56 and 0.5 mM, respectively. Interestingly, in some experiments, a transient burst of $[Ca^{2+}]_i$ spikes was seen superimposed on the background $[Ca^{2+}]_i$ rise (*e.g. islets 2* and *4* in Fig. 1*B*).

Lowering glucose concentration to 3 mM is well known to hyperpolarize the β-cell membrane and suppress the electrical activity (25). This is accompanied by the cessation of the $[Ca^{2+}]_i$ oscillations and by a fall in $[Ca^{2+}]_i$ (Fig. 2*A*). Raising the $[Ca^{2+}]_o$ to 12.8 mM in the presence of 3 mM glucose evoked a pronounced $[Ca^{2+}]_i$ rise, which in the experiment shown in Fig. 2*A* consisted of a rapid peak followed by decay toward a plateau. Other islets bathed in 3 mM glucose (*e.g. islets 1* through *3* in Fig. 2*B*) displayed a variety of response patterns to high Ca^{2+}, apparently indistinguishable from those displayed in the presence of 11 mM glucose and nifedipine. Control experiments showed that the presence of nifedipine did not change the characteristics of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises recorded in the presence of 3 mM glucose (*islets 4* through *6*). In these experiments, the difference between the $[Ca^{2+}]_i$ recorded 60 s after exposure to high Ca^{2+} and basal $[Ca^{2+}]_i$ averaged 87 ± 58 nM (n = 7 different islets) and 88 ± 46 nM (n = 9 islets) in the absence and presence of nifedipine, respectively.

High Ca^{2+} raised the $[Ca^{2+}]_i$ in a dose-dependent fashion (Fig. 3*A*). Since, in several experiments, the $[Ca^{2+}]_i$ did not reach a maximum within 3–4 min after exposure to high Ca^{2+} solutions, the extent of the $[Ca^{2+}]_i$ rise was assessed by taking the difference between the level measured 60 s after the beginning of each pulse and the basal $[Ca^{2+}]_i$ measured immediately before the pulse. This response was plotted against the size of the high Ca^{2+} pulses in Fig. 3*B*, yielding an average EC_{50} value ($[Ca^{2+}]_o$ necessary to achieve half-maximal $[Ca^{2+}]_i$ rises) of 3.6 mM.

The high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises can conceivably be mediated by enhanced Ca^{2+} influx or by enhanced Ca^{2+} release from internal stores. We have investigated whether Ins 1,4,5-P_3-sensitive Ca^{2+} stores might be involved by assessing the response to high Ca^{2+} after extensive depletion of these stores.

In pancreatic β-cells, acetylcholine and other muscarinic receptor agonists raise the $[Ca^{2+}]_i$, at least in part, by triggering the release of Ca^{2+} from Ins 1,4,5-P_3-sensitive stores (26–28). Fig. 4*A* shows that, in the presence of nifedipine, whole pancreatic islets respond to acetylcholine with a sharp $[Ca^{2+}]_i$ rise followed by a relaxation toward baseline.[2] Subsequent exposure to a Ca^{2+}-free medium supplemented with EGTA caused a pronounced $[Ca^{2+}]_i$ fall. Depletion of Ins 1,4,5-P_3-sensitive Ca^{2+} stores was achieved by exposing the islets twice to acetylcholine in Ca^{2+}-free medium, as evidenced by the almost complete lack of response to the second acetylcholine pulse (Fig. 4*A*). Under these conditions, challenging the islets with 12.8 mM Ca^{2+} caused a pronounced and rapid $[Ca^{2+}]_i$ rise, the amplitude of which was about 65% (average 45 ± 28%, n = 3 islets) greater

[2] In the absence of nifedipine, the typical $[Ca^{2+}]_i$ response to muscarinic agonists is a rapid increase, followed by a decrease toward a plateau (data not shown, but see Refs. 26 and 27 for experiments carried out in isolated β-cells).

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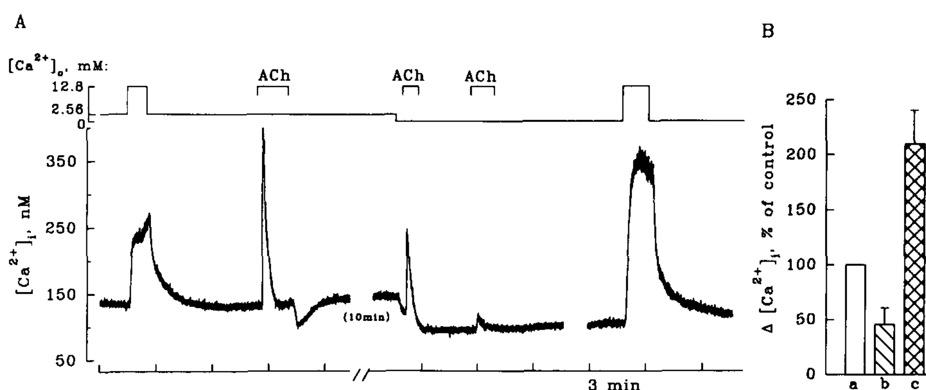

FIG. 4. **Effect of depleting the acetylcholine-mobilizable internal Ca^{2+} pools on the amplitude of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise.** *A*, the islet was exposed to brief pulses of 12.8 mM Ca^{2+} and 10 μM acetylcholine (*ACh*), as depicted in the *upper part* of the figure. The Ca^{2+}-containing solution was then replaced for a Ca^{2+}-free solution supplemented with 0.5 mM EGTA, and the islet was briefly exposed to 10 μM acetylcholine (two consecutive pulses) in the virtual absence of extracellular Ca^{2+}. The islet was finally challenged with a brief pulse of 12.8 mM Ca^{2+} after the acetylcholine-mobilizable internal Ca^{2+} pool had been extensively depleted, as assessed by the strong attenuation of the acetylcholine effect. The perifusion solution contained 3 mM glucose and 10 μM nifedipine throughout the experiment. *B*, the difference ($\Delta[Ca^{2+}]_i$) between the $[Ca^{2+}]_i$ recorded 60 s after the start of the high Ca^{2+} (12.8 mM) pulse and the $[Ca^{2+}]_i$ recorded immediately prior to the pulse in the virtual absence of extracellular Ca^{2+} (*column labeled c*) was normalized to the response obtained when the $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM (*column labeled a*; absolute response was 115 ± 22 nM, *n* = 3 islets). Also represented for comparison is the difference between the steady-state $[Ca^{2+}]_i$ levels recorded in 2.56 mM Ca^{2+} and 0 Ca^{2+} (*column labeled b*). The data were obtained from three experiments identical with that illustrated in *panel A* (*vertical bars* represent ±S.D.).

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than the sum of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise observed under control conditions and the $[Ca^{2+}]_i$ fall subsequent to external Ca^{2+} removal (Fig. 4*B*).

The high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises can be interpreted assuming an enhanced Ca^{2+} entry through either selective or nonselective cation channels. Ni^{2+} has been reported to block Ca^{2+} translocation through voltage-sensitive Ca^{2+} channels, receptor-operated Ca^{2+} channels, and cation channels linked to the refilling of internal Ca^{2+} pools (29–39). We have assessed the sensitivity of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises to Ni^{2+} concentrations in the millimolar range. Fig. 5*A* shows that 3 mM Ni^{2+} reduced the amplitude of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises recorded in the presence of 3 mM glucose and nifedipine by approximately 86%. The average reduction of these transients was 70 ± 18% (*n* = 4 different islets; Fig. 5*B*). Preliminary experiments indicated that 0.5 mM Ni^{2+} was ineffective, while 2 mM Ni^{2+} reduced the amplitude of the transients by approximately 45% (data not shown).

Examination of the fluorescence changes at the individual excitation wavelengths revealed that high Ca^{2+} increased and decreased the fluorescence recorded at 340 (F_{340}) and 380 nm (F_{380}), respectively, as expected from the known spectroscopic behavior of fura-2 (Fig. 5*C*). The immediate effect of adding 3 mM Ni^{2+} was to promote symmetrical changes on F_{340} and F_{380}, resulting in a slight and transient $[Ca^{2+}]_i$ rise, similar to that shown in Fig. 5*A*. Subsequently, Ni^{2+} decreased F_{340} and F_{380} in a manner compatible with the divalent cation permeating the membrane and quenching fura-2 fluorescence (32, 40, 41). The quenching effects of Ni^{2+} were canceled out by the ratio F_{340}/F_{380} and, hence, had no artifactual expression on the calibrated $[Ca^{2+}]_i$ traces (Fig. 5*A*). Further elevation of $[Ca^{2+}]_o$ in the continued presence of Ni^{2+} evoked attenuated symmetrical fluorescence changes at 340 and 380 nm (Fig. 5*C*).

The anion channel blocker diphenylamine-2-carboxylate (DPC or *N*-phenylanthranilic acid) and related compounds (42–46) have been reported to block a nonselective cation channel present in several cell types (47–52). We have assessed the sensitivity of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise to DPC.

Fig. 6 shows that DPC is an effective blocker of the latter $[Ca^{2+}]_i$ rises. Indeed, superfusing the islets with 350 μM DPC in the continued presence of 11 mM glucose, nifedipine and 12.8 mM Ca^{2+} rapidly reduced the $[Ca^{2+}]_i$ to a level slightly below the

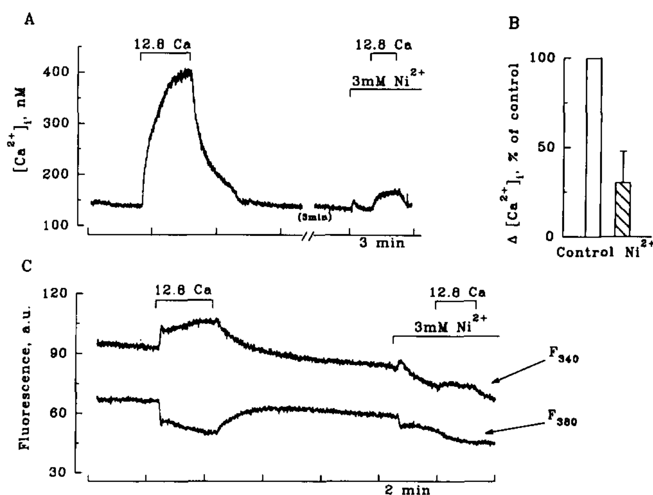

FIG. 5. **The high Ca^{2+}-evoked $[Ca^{2+}]_i$ transient is inhibited by millimolar concentrations of Ni^{2+}.** *A*, the islet was exposed to brief pulses of 12.8 mM Ca^{2+} in the absence and presence of 3 mM Ni^{2+}, as indicated by the *brackets* (basal $[Ca^{2+}]_o$ was 2.56 mM). The perifusion solution contained 3 mM glucose and 10 μM nifedipine. *B*, the difference ($\Delta[Ca^{2+}]_i$) between the $[Ca^{2+}]_i$ recorded 60 s after the start of the high Ca^{2+} (12.8 mM) pulse and the $[Ca^{2+}]_i$ recorded immediately prior to the pulse in the presence of 3 mM Ni^{2+} (*column labeled Ni^{2+}*) was normalized to the response obtained in the absence of Ni^{2+} (*column labeled control*; absolute response was 138 ± 88 nM, *n* = 4 islets). The data were obtained from four experiments identical with that illustrated in *panel A* (*vertical bar* represents ±S.D.). *C*, effect of the experimental maneuvers shown in *panel A* on the time course of the fluorescence recorded at 340 (F_{340}) and 380 nm (F_{380}) from a different islet. The continuous drop in fluorescence is due to dye photobleaching. The enhanced fluorescence decay observed in the presence of Ni^{2+} is attributable to quenching of fura-2 fluorescence by cytoplasmic Ni^{2+}.

baseline (Fig. 6*A*). In several experiments (*e.g.* that depicted in Fig. 6*A*), DPC caused the delayed appearance of high frequency $[Ca^{2+}]_i$ spikes, an effect that was eventually accompanied by a pronounced secondary increase toward higher levels. The appearance of these $[Ca^{2+}]_i$ spikes was observed in 61% of the experiments (8 out of 13) in which DPC was applied in the presence of 11 mM glucose and nifedipine. In contrast, the secondary effect of DPC was never observed in cells hyperpolar-

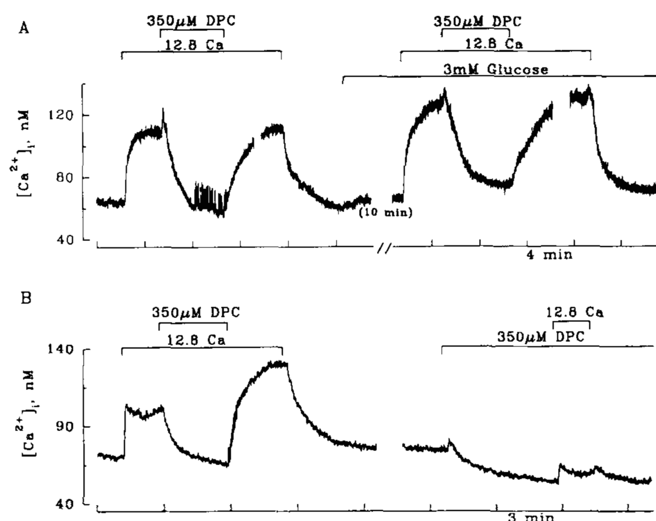

FIG. 6. **The nonselective cation channel blocker diphenylamine-2-carboxylate blocks the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises.** *A*, the $[Ca^{2+}]_o$ was temporarily raised from 2.56 to 12.8 mM in the continued presence of 11 mM glucose (*left*). Diphenylamine-2-carboxylate (DPC) was temporarily applied during the period of exposure to high Ca^{2+}, as indicated by the *bracket*. The glucose concentration in the perfusate was subsequently decreased to 3 mM, after which the islet was again subjected to DPC in the presence of 12.8 mM external Ca^{2+} (*right*). The solution contained 10 μM nifedipine throughout. Note that the inhibitory effect of DPC in 3 mM glucose is less pronounced than in 11 mM glucose. This experiment is representative of 7 similar experiments. *B*, DPC was temporarily applied to a different islet in the presence of 12.8 mM external Ca^{2+} (*left*), following an experimental protocol identical with that shown in *panel A*. The islet was then incubated with DPC and challenged with high Ca^{2+} in the presence of the drug (*right*). The solution contained 11 mM glucose and 10 μM nifedipine throughout. This experiment is representative of five similar experiments.

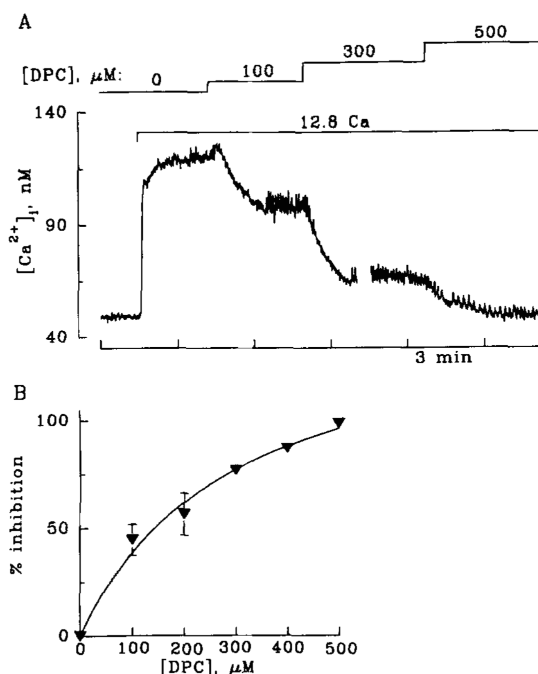

FIG. 7. **DPC suppresses the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise in a dose-dependent fashion.** *A*, the $[Ca^{2+}]_o$ was raised from 0.2 to 12.8 mM in the presence of 11 mM glucose and 10 μM nifedipine. The islet was subsequently challenged with increasing diphenylamine-2-carboxylate (*DPC*) concentrations in the continued presence of high Ca^{2+}, as denoted in the *upper diagram*. *B*, data pooled from experiments similar to that shown in the *upper panel*. The DPC-mediated inhibition of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise was assessed using the ratio $R = 100\ (C_p - C_i)/(C_p - C_b)$, where C_i is the steady-state $[Ca^{2+}]_i$ at each DPC concentration, C_p is the peak $[Ca^{2+}]_i$ recorded prior to exposure to 100 μM DPC and C_b stands for the basal $[Ca^{2+}]_i$. The ratio *R* (% inhibition) was plotted against DPC concentration and was fitted to a saturation function. The *vertical bars* represent ± S.D. (n = 3–6 different islets; the size of the *bars* did not exceed the size of the *symbols* in the range 300–500 μM). Absolute high Ca^{2+}-evoked $[Ca^{2+}]_i$ response ($C_p - C_b$): 64 ± 16 nM (n = 8 islets).

ized by exposure to 3 mM glucose (n = 5 islets, an example of which is shown to the *right* of Fig. 6*A*). The blocking effect of DPC was readily reversible, as assessed by the prompt $[Ca^{2+}]_i$ return toward extrapolated pre-DPC levels observed upon removal of the blocker (Fig. 6*A*). Interestingly, DPC was somewhat less effective in impairing the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise observed in the presence of 3 mM glucose than that observed in cells depolarized by exposure to 11 mM glucose. Thus, whereas under the latter condition 350 μM DPC caused the $[Ca^{2+}]_i$ to drop slightly below baseline, in the presence of 3 mM glucose the steady-state $[Ca^{2+}]_i$ recorded during DPC exposure consistently remained above baseline (Fig. 6*A*). DPC appeared to block the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises about as efficiently when applied in the presence of elevated external Ca^{2+} levels than when applied prior to the high Ca^{2+} pulse (Fig. 6*B*). In addition, Fig. 6*B* shows that DPC was effective in lowering the $[Ca^{2+}]_i$ at standard extracellular Ca^{2+} concentrations (*i.e.* 2.56 mM).

Fig. 7*A* shows that DPC blocked the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises produced in the presence of 11 mM glucose and nifedipine in a dose-dependent fashion. The IC_{50} value calculated from these experiments was 145 μM (Fig. 7*B*).

Since glucose is the major physiological secretagogue of the pancreatic β-cell, we have carried out experiments designed to investigate the modulation of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises by glucose metabolism.

Raising the glucose concentration from 3 to 30 mM evoked a sharp rise in $[Ca^{2+}]_i$, which returned to basal levels after switching the glucose concentration back to 3 mM (Fig. 8*A*). The glucose-evoked $[Ca^{2+}]_i$ transient originated from Ca^{2+} entry through L-type Ca^{2+} channels, since raising the glucose concentration in the presence of nifedipine actually caused a fall in $[Ca^{2+}]_i$ (this fall was probably caused by the stimulation of Ca^{2+} sequestering/extrusion mechanisms, as previously hypothesized (53–55)). Comparison of the effects of high Ca^{2+} pulses in the presence of substimulatory (3 mM) and suprastimulatory (30 mM) glucose concentrations (nifedipine throughout) is also shown in Fig. 8*A*. Thus, raising the $[Ca^{2+}]_o$ in the presence of 30 mM glucose produced a $[Ca^{2+}]_i$ transient with a somewhat diminished amplitude and attenuated rate of rise relative to that obtained in the presence of 3 mM glucose. Data gathered from experiments similar to that depicted in Fig. 8*A*, but in which the perifusion solution containing 3 mM glucose was replaced for a glucose-free solution, showed that the amplitudes of the $[Ca^{2+}]_i$ rises measured at different time points after delivering the high Ca^{2+} pulse in the presence of 30 mM glucose amounted to 80–90% of those measured in 0 glucose (Fig. 8*B*). In keeping with these data, we have also observed a $[Ca^{2+}]_i$ rise following glucose deprivation in the continued presence of nifedipine and 12.8 mM external Ca^{2+} (Fig. 8*C*). This rise was sustained within the first 3–5 min of exposure to the glucose-free solution, but usually faded throughout longer incubations (data not shown).

Fig. 9*A* depicts an experiment where an islet was challenged with high Ca^{2+} in the absence and presence of the glycolytic inhibitor mannoheptulose (11 mM glucose and nifedipine throughout). In this experiment, the $[Ca^{2+}]_i$ transient consisted of a rapid and transient rise, followed by a slower secondary rise. Whereas the initial component remained practically unaffected by mannoheptulose, the slower component was somewhat increased by the glycolytic inhibitor. However, this difference was not evident when the amplitudes of the $[Ca^{2+}]_i$ rises

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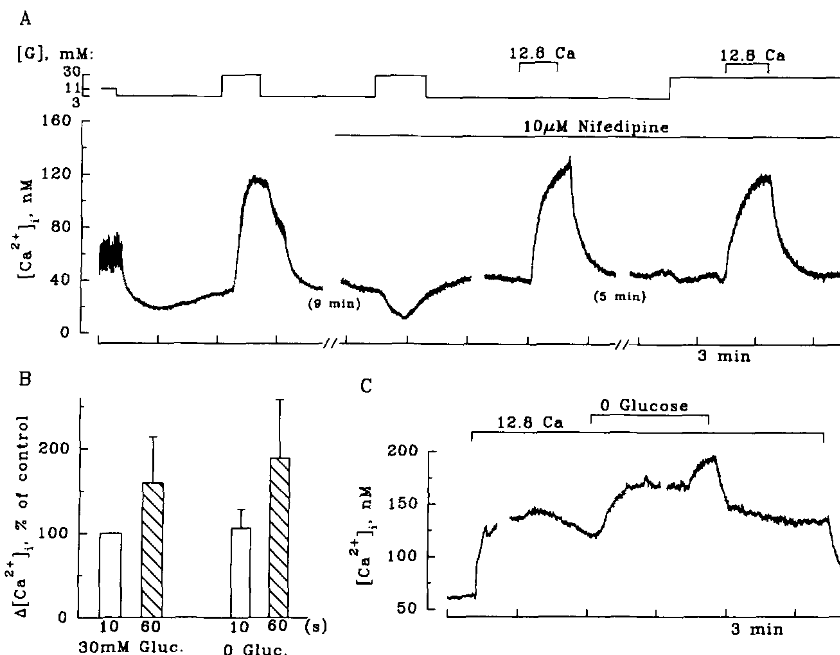

FIG. 8. **Glucose dependence of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise.** *A*, the glucose concentration in the perfusate (initially 11 mM) alternated between 3 mM and 30 mM as depicted in the *upper diagram*. The right part of the experiment was carried out in the continued presence of nifedipine, as denoted by the *bar*. The $[Ca^{2+}]_o$ was raised from 2.56 to 12.8 mM, as denoted by the *brackets*, in the continued presence of nifedipine and either 3 mM or 30 mM glucose. *B*, the relative magnitudes of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ responses obtained in low and high glucose were determined from experiments similar to that depicted in *panel A*, except that the solution containing 3 mM glucose was replaced for a glucose-free solution, and the $[Ca^{2+}]_o$ was raised to 12.8 mM from a lower level (0.5 mM). The differences ($\Delta[Ca^{2+}]_i$) between the $[Ca^{2+}]_i$ recorded at different time points (10 and 60 s) after the start of the high Ca^{2+} pulse and the $[Ca^{2+}]_i$ recorded immediately prior to the pulse, either in the presence of 30 mM glucose (*two leftmost columns*) or in the absence of glucose (*two rightmost columns*), were normalized to the response obtained 10 s after the delivery of the high Ca^{2+} pulse in 30 mM glucose (absolute response was 27 ± 8 nM, n = 6 islets). The *vertical bars* represent ± S.D. (n = 4–6 different islets). Nifedipine (10 μM) was present throughout the experiments. Incubation time in 0 or 30 mM glucose prior to delivery of the high Ca^{2+} pulses was 10–12 min. *C*, glucose was temporarily withdrawn from a solution containing 11 mM glucose, in the continued presence of 12.8 mM external Ca^{2+}, as indicated by the *upper bracket*. Basal $[Ca^{2+}]_o$ before and after exposure to the high Ca^{2+} pulse: 2.56 mM. The solution contained 10 μM nifedipine throughout.

measured at different time points after delivering the high Ca^{2+} pulse were pooled from a larger group of experiments, as shown in Fig. 9*B*. Exposure to 10–20 mM mannoheptulose in the continued presence of nifedipine, 11 mM glucose, and 12.8 mM Ca^{2+} mimicked the effect of glucose deprivation depicted in Fig. 8*C*. Interestingly, in these experiments, the mannoheptulose-evoked $[Ca^{2+}]_i$ rise was over in less than 8 min (incubation time with mannoheptulose in the experiments depicted in Fig. 9*B* was 10–12 min).

DISCUSSION

We have found that whole pancreatic islets respond to stepwise increases in extracellular Ca^{2+} concentration with robust $[Ca^{2+}]_i$ rises in the presence of the L-type Ca^{2+} channel blocker nifedipine. It is noteworthy that the dihydropyridine suppressed the $[Ca^{2+}]_i$ oscillations recorded in the presence of 11 mM glucose and lowered the $[Ca^{2+}]_i$ in a manner compatible with the complete blockade of β-cell spiking activity (5, 19). Thus, the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises originate from a process unrelated to Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels.

Nilsson *et al.* (12) have hypothesized that Ca^{2+} release from Ins 1,4,5-P_3-sensitive internal Ca^{2+} stores mediates the $[Ca^{2+}]_i$-raising action of external Ca^{2+} on β-cells isolated from obese hyperglycemic mice. Using whole islets of Langerhans isolated from normal mice, we have obtained robust and rapid $[Ca^{2+}]_i$ rises after extensive depletion of acetylcholine-mobilizable internal Ca^{2+} pools (this depletion was achieved by repetitively challenging the islets with acetylcholine in the presence of EGTA-containing Ca^{2+}-free solutions). Even assuming that Ca^{2+} release from newly refilled Ca^{2+} stores might ultimately contribute to the $[Ca^{2+}]_i$ rise, some Ca^{2+} influx would have to refill these stores and, thus, be expected to contribute to the $[Ca^{2+}]_i$ transient. It could nonetheless be argued that a significant fraction of the $[Ca^{2+}]_i$ transient might originate from internal Ca^{2+} pools distinct from the Ins 1,4,5-P_3-sensitive stores involved in the acetylcholine action. Although we cannot entirely rule out this possibility, the fact that prolonged incubations with EGTA-containing Ca^{2+}-free solutions apparently cause a global depletion of internal Ca^{2+} pools, together with the observation that two different cation channel blockers, Ni^{2+} and DPC, exert pronounced inhibitory effects on the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise (see below), strongly suggest that this is not the case. We therefore propose that the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise is primarily accounted for by Ca^{2+} influx through dihydropyridine- and voltage-insensitive channels, a hypothesis consistent with the observation that the β-cell undergoes a pronounced depolarization when challenged with high Ca^{2+} in the presence of 11 mM glucose and nifedipine (5, 19).

Our experiments show that the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises are not critically dependent on whether the islets were exposed to high Ca^{2+} in the presence of low (*i.e.* 0 or 3 mM) or intermediate-high (*i.e.* 11 or 30 mM) glucose concentrations. The β-cell membrane is about 25 mV more depolarized in the presence of 11 mM glucose and nifedipine than in 3 mM glucose.[3] Thus, the

[3] When applied to microdissected mouse islets in the presence of 11 mM glucose, submicromolar nifedipine concentrations change bursting electrical activity into a pattern of continuous spiking activity at the burst plateau level (56). We have also observed that micromolar nifedipine concentrations depolarize β-cells to the plateau level, an effect that is accompanied by the complete suppression of spiking activity (R. M. Barbosa, R. M. Santos, and L. M. Rosário, unpublished observations) and closely resembles the published effects of the dihydropyridine in the presence of 11 mM glucose and tolbutamide (5). Since the membrane

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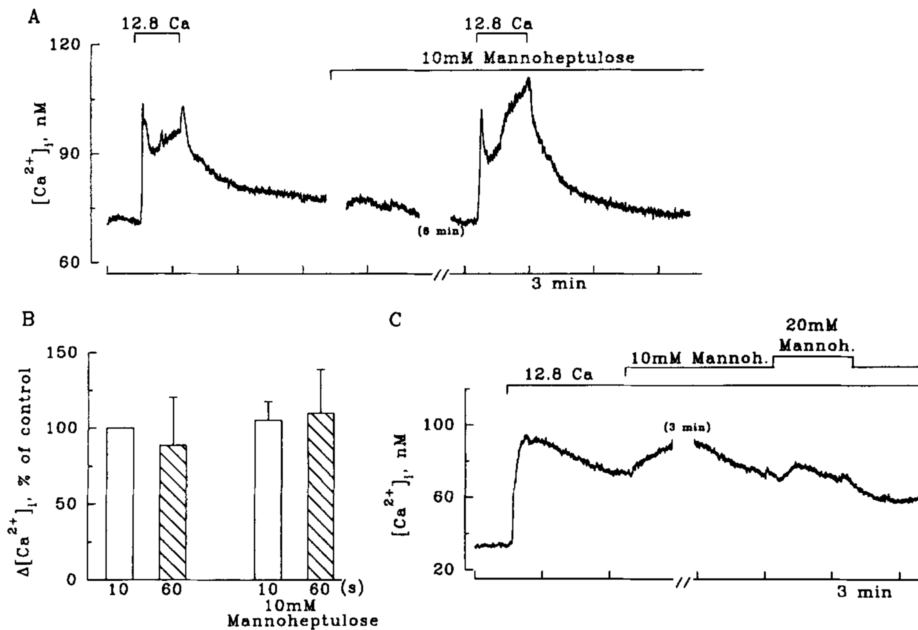

FIG. 9. **Modulation of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rise by the glycolytic inhibitor mannoheptulose.** *A*, the $[Ca^{2+}]_o$ was raised from 0.5 to 12.8 mM in the absence (*left*) and presence (*right*) of mannoheptulose, as indicated by the *square brackets*. The solution contained 11 mM glucose and 10 μM nifedipine throughout the experiment. *B*, the relative magnitudes of the high Ca^{2+}-evoked $[Ca^{2+}]_i$ responses obtained in the presence and absence of mannoheptulose were determined from experiments similar to that depicted in *panel A*. The differences ($\Delta[Ca^{2+}]_i$) between the $[Ca^{2+}]_i$ recorded at different time points (10 and 60 s) after the start of the high Ca^{2+} pulse and the $[Ca^{2+}]_i$ recorded immediately prior to the pulse, either in the absence (*two leftmost columns*) or in the presence (*two rightmost columns*) of 10 mM mannoheptulose, were normalized to the response obtained 10 s after the delivery of the high Ca^{2+} pulse in the absence of mannoheptulose (absolute response was 21 ± 7 nM, $n = 6$ islets). The *vertical bars* represent ± S.D. ($n = 6$ different islets). Nifedipine (10 μM) was present throughout the experiments. Incubation time in mannoheptulose was 10–12 min in all cases. *C*, mannoheptulose (10 and 20 mM) was temporarily added to the perifusion solution in the continued presence of 12.8 mM external Ca^{2+}, as indicated in the *upper diagram*. Basal $[Ca^{2+}]_o$ before exposure to the high Ca^{2+} pulse: 2.56 mM. The solution contained 11 mM glucose and 10 μM nifedipine throughout.

Ca^{2+} translocation mechanism underlying the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises appears to be largely insensitive to membrane potential in the physiological range (−65/−40 mV). The slight potentiation of the high Ca^{2+} response obtained in the presence of low glucose concentrations may conceivably be due to the fact that glucose metabolism increases the Ca^{2+} buffering capacity of the β-cell (57), to the increased transmembrane electrical gradient (assuming that Ca^{2+} flows through a cation channel, see below), or to both factors.

We have shown that millimolar concentrations of Ni^{2+} reduce the size of the $[Ca^{2+}]_i$ rise, presumably by competing with external Ca^{2+} for access to the cytoplasm through the same channel. In fact, since Ni^{2+} is an effective quencher of fura-2 fluorescence (32, 40, 41), the observed quenching effect (Fig. 5*C*) is indicative of a significant influx rate of the cation. Besides blocking voltage-sensitive Ca^{2+} channels, Ni^{2+} is well known to block, albeit with different potencies, various types of voltage-insensitive cation channels which are directly or indirectly linked to the activation of plasma membrane receptors (*e.g.* receptor-operated Ca^{2+} channels and channels activated by the emptying of internal Ca^{2+} stores) (29–39). Since pancreatic β-cells are endowed with membrane receptors for various hormones and neurotransmitters liberated from nerve terminals within the islet and capable of discharging the latter stores (*e.g.* α_1-adrenergic, muscarinic, purinergic, and cholecystokinin receptors), it is conceivable that background hormone and/or neurotransmitter release might cause the sustained activation of Ca^{2+}-conducting cation channels and that these, in turn, might underlie the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises. However, we have observed recently that isolated β-cells respond to Ca^{2+} pulses in a manner entirely analogous to whole islets. Specifically, the high Ca^{2+} responses observed in these cells retain the sensitivity to Ni^{2+} and DPC demonstrated here for whole islets.[4] Thus, the available evidence indicates that the $[Ca^{2+}]_i$ rises described in the present study reflect an intrinsic property of pancreatic β-cells.

Another class of voltage-insensitive cation channels, the Ca^{2+}-activated nonselective cation channel, has been described in many different cell types and may, in fact, be ubiquitous among mammalian cells (58). Interestingly, a Ca^{2+}-activated nonselective cation channel with 20–25 pS unitary conductance has been characterized in excised patches from rat and human insulinoma cells (59–61), but its Ca^{2+} permeability and possible occurrence in normal pancreatic β-cells have not yet been determined. The range of Ca^{2+} concentrations required for the activation of the latter channel in excised patches is well above physiological levels (61), thus making it a seemingly unattractive candidate for the mediation of physiologically relevant processes in β-cells (62). However, since Ca^{2+}-activated nonselective cation channels from other cells appear to exhibit a "physiological" sensitivity to $[Ca^{2+}]_i$ when present in intact cells (63), the poor sensitivity to Ca^{2+} revealed by the channels in excised patches may represent an artifact related to the loss of labile cytoplasmic components during patch excision, as discussed by Petersen (64). In this respect, it is noteworthy that unidentified active cation channels with an unitary conductance similar to that found in insulinoma cells have been reported to occur in cell-attached patches of β-cell membranes (65–67).

potential at the burst plateau phase is typically −35/−40 mV (25), β-cells are at least 25 mV more depolarized in the presence of 11 mM glucose and nifedipine than in 3 mM glucose (membrane potential approximately −65 mV (25)).

[4] A. M. Silva, A. P. Salgado, R. M. Santos, and L. M. Rosário, manuscript in preparation.

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The possible involvement of nonselective cation channels in the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises reported in this study is also emphasized by the exquisite sensitivity of these rises to diphenylamine-2-carboxylate. Indeed, although this and related compounds are well known anion channel blockers (42–46), they have been reported to block nonselective cation channels in different cell types (47–52). DPC blocks most of the activity of the latter channels at 100 μM (49), which compares well with the IC_{50} for the DPC block reported in this study (145 μM). Interestingly, DPC is an effective blocker of high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises in the mouse mandibular cell line ST_{885} (68, 69), where DPC-sensitive nonselective cation channels have also been reported (49). Our study emphasizes two aspects of the action of DPC that, to our knowledge, have remained undetected in previous studies. First, in a significant number of experiments, fast $[Ca^{2+}]_i$ spikes probably associated with action potentials became evident after DPC treatment in the presence of high Ca^{2+}, 11 mM glucose, and nifedipine. This suggests that DPC might either react with the free dihydropyridine or somehow interfere with its binding site on the Ca^{2+} channel, thereby removing the block. (This possible interference is probably devoid of major consequences as far as the analysis of the DPC effect on the $[Ca^{2+}]_i$ rises is concerned, since in most experiments the appearance of the $[Ca^{2+}]_i$ spikes was not accompanied by a progressive $[Ca^{2+}]_i$ rise.) Secondly, the DPC binding to the putative cation channel appeared to be voltage-dependent, since DPC treatment was somewhat less effective in suppressing the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises in cells hyperpolarized by exposure to low glucose than in cells exposed to 11 mM glucose. A similar voltage dependence has been reported for the quinine block of nonselective cation channels in a mandibular cell line (49).

A nonspecific cation channel provided with Ca^{2+}-conducting properties (G-channel) has been previously described in human pancreatic β-cells and chromaffin cells (70, 71). While the activity of the G-channel appears to be predominantly expressed in the presence of stimulatory concentrations of glucose (70), the putative channel proposed in the present study is relatively insensitive to glucose metabolism, as indicated by the fact that the high Ca^{2+}-evoked $[Ca^{2+}]_i$ rises are poorly sensitive to changes in glucose concentration (see above) and to the glycolytic inhibitor mannoheptulose (a competitive blocker of glucokinase). In keeping with the idea that β-cells are endowed with glucose-activated cation channels, there have been previous contentions that these cells are provided with a voltage-insensitive modality of glucose-evoked Ca^{2+} influx (72). However, our experiments show that the glucose-evoked $[Ca^{2+}]_i$ rises can be totally blocked by nifedipine (Fig. 8*A*). This observation indicates that glucose-induced Ca^{2+} influx is exclusively supported by voltage-sensitive Ca^{2+} channels in mouse β-cells.

In summary, we have provided evidence that the pancreatic β-cell has a high background Ca^{2+} influx rate across its membrane in the absence of functional voltage-sensitive Ca^{2+} channels. This influx appears to be supported by a Ni^{2+}- and DPC-blockable nonselective cation channel. The basal leakiness of the β-cell membrane to Ca^{2+} is especially apparent from the exquisite sensitivity of the resting $[Ca^{2+}]_i$ to both changes in $[Ca^{2+}]_o$ around the physiological levels and to acute DPC applications at standard external Ca^{2+} levels. Not withstanding an important role for the putative nonselective cation channel in the control of β-cell membrane potential or in other cellular functions, this channel does not appear to be critically affected by glucose metabolism and, thus, may be vital to supply β-cells with Ca^{2+} in the absence of stimulatory levels of fuel secretagogues.

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