Characterization of Voltage-Dependent Ca^{2+} Channels in β-Cell Line

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Although there is compelling pharmacological evidence based on Ca^{2+}-channel antagonist studies suggesting that the voltage-dependent Ca^{2+} channels regulate insulin release, no direct comparison with Ca^{2+} currents exists. This is particularly important because of the recent demonstration in other cell types of one and possibly two Ca^{2+} channels that are insensitive to Ca^{2+}-channel antagonists, the dihydropyridines and the phenylalkylamines. Using an SV40-transformed pancreatic β-cell line (HIT cells), we determined how voltage-dependent Ca^{2+} channels are involved in stimulus-secretion coupling. Ca^{2+} currents were measured with the tight-seal technique for whole-cell recording. The cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) was followed with the fluorescent probe Fura 2, and the measurements were compared with insulin secretion stimulated by depolarizing the cells with K^+. The Ca^{2+} current contained two components: a rapidly decaying current activated at −50 to −40 mV that decayed with a time constant of 25 ms and a very slowly decaying component activated at −40 mV. Both components were sensitive to the Ca^{2+}-channel antagonist nimodipine. There is excellent agreement in the concentration of nimodipine that inhibited Ca^{2+} current and the increase in $[Ca^{2+}]_i$ in response to K^+ depolarization (IC_{50} of 15 and 6 nM, respectively). Nimodipine inhibited insulin release over a similar dose-response range with an IC_{50} of 1.5×10^{-9} M. These studies indicate that the increase in $[Ca^{2+}]_i$ in response to β-cell depolarization can be accounted for by the influx of this ion through a single class of dihydropyridine-sensitive Ca^{2+} channels in the cell membrane. *Diabetes* 38:188–93, 1989

With the development of fluorescent Ca^{2+} indicators and insulin-secreting cell lines, the cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) can be quantitated in β-cells, clarifying the role of this second messenger in regulating insulin secretion. Studies have shown that depolarizing concentrations of K^+ (1,2), the amino acids alanine and leucine (3), or sulfonylureas (4) activate voltage-dependent Ca^{2+} channels to produce Ca^{2+} influx down the electrochemical gradient into the β-cell. The rise in $[Ca^{2+}]_i$ triggers a monophasic pattern of insulin release, even in a glucose-free environment. Furthermore, the organic Ca^{2+}-channel antagonists inhibit insulin secretion and the rise in $[Ca^{2+}]_i$ in parallel in a concentration-dependent reversible manner (5). These studies clearly establish a pivotal role for the voltage-gated Ca^{2+} channel in regulating insulin release.

Although reports have described Ca^{2+} currents in cultured neonatal rat islet cells (6), RINm5F cells (7), and NMRI mouse islet cells (8), no direct examination has been made of the concentration response of the currents to Ca^{2+}-channel antagonists for comparison with the effects on intracellular Ca^{2+} and insulin secretion. This is important because of the recent demonstration of at least one and possibly two Ca^{2+} channels that do not show dihydropyridine sensitivity (9–12). A recent report suggests the presence of one of these Ca^{2+} channels in rat β-cells and in HIT cells (13).

We used the patch-clamp technique for whole-cell recording to examine Ca^{2+} currents through Ca^{2+} channels to determine at what voltages the channels open, how long they stay open, and the sensitivity of the channels to the Ca^{2+} antagonist nimodipine. By comparing whole-cell voltage recordings with changes in insulin secretion and $[Ca^{2+}]_i$, it was possible to show that all of the Ca^{2+} current enters the HIT cell through a dihydropyridine-sensitive class of Ca^{2+} channels.

MATERIALS AND METHODS

Whole-cell current recording. The tight-seal technique was used to record whole-cell currents as described by Hamill et al. (14). Resting membrane potentials and action poten-

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tials were studied with extracellular recording solutions containing (in mM) 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, and the pH was adjusted to 7.3 with NaOH. The pipette (intracellular solution) contained (in mM) 140 KCl, 10 HEPES, and 2 $MgCl_2$, and the pH was adjusted to 7.3 with KOH. Whole-cell Ca^{2+} currents were measured under voltage clamp with an extracellular solution containing (in mM) 140 tetraethylammonium chloride, 2.0 $CaCl_2$, 5.4 KCl, 2 glucose, 10 HEPES, and 5.0 4-aminopyridine, and the pH was adjusted to 7.4 with KOH. The intracellular pipette solution contained (in mM) 105 *N*-methylglucamine, 15 1,2 *bis*-aminophenoxyethane-*N,N,N'*, *N'*-tetraacetic acid, 10 HEPES, 110 L-aspartic acid, and 2.5 $MgCl_2$, and the pH was adjusted to 7.2 with NMDG. In some experiments, 2 mM $BaCl_2$ replaced the 2 mM $CaCl_2$ in the extracellular solution. Chemicals were obtained from Sigma (St. Louis, MO) and BDH Chemicals (Poole, UK). All studies were performed at room temperature.

The pipettes were made with 8161 or 7052 glass (Garner Glass). Pipettes were prepared by a two-stage pull and fire polished. The resistance of the pipettes ranged from 3 to 5 MΩ. The indifferent electrode was an Ag-AgCl plug connected to the bath via a 200-mM KCl bridge. The nimodipine was applied to the cell from a micropipette with pressure application (Picospritzer-General Valve) or added directly to the bath. Similar results were obtained with both methods. A stock solution of 1 mM nimodipine was prepared in polyethylene glycol, and subsequent dilutions were made in the extracellular solution. Nimodipine was a gift from A. Scriabine (Miles, Elkhart, IN). Voltage and current data for whole-cell experiments were digitized on line and stored for subsequent computer analysis. Where specified, the currents were leakage corrected from appropriately scaled pulses.

Insulin secretion. HIT cells were plated into 12-well Costar plates at a concentration of 5 × 10^5 cells/25-mm well and grown for 3 days. Growth medium RPMI-1640 was then removed, and insulin secretion from HIT cell monolayers during a 10-min stimulation period was determined as previously described (2,15,16).

Measurement of $[Ca^{2+}]_i$ with Fura 2. The $[Ca^{2+}]_i$ was measured with Fura 2 by conventional means as described by Grynkiewicz et al. (17). HIT cells were suspended in Krebs-Ringer bicarbonate buffer containing (in mM) 20 HEPES, 1.5 $CaCl_2$, 4 glucose, and 0.1% bovine serum albumin (BSA). Fura 2 loading was achieved by incubating the suspended cells with the permeable precursor of Fura 2, Fura 2AM, to a final concentration of 10^{-6} M at 37°C for 30 min. The Fura 2AM ester enters the cells and is hydrolyzed to the impermeable Fura 2, which remains trapped in the cytosol, and binds intracellular Ca^{2+}. Unincorporated Fura 2AM was removed by washing the cells and resuspending them in fresh buffer. Fluorescence measurements of suspended cells (1 × 10^6/ml) in a quartz cuvette were performed with a Perkin-Elmer LS5 spectrophotometer. Test agents were added from concentrated solutions. With a computer program (courtesy D. Brown, Perkin-Elmer), excitation wavelengths alternated between 340 and 380 nm, and the emission was measured at 505 nm. The fluorescence measurements were performed at 20°C to minimize dye leakage from the cells. Changes in the 340-to-380 fluorescence ratio were recorded and stored. Calibration was performed by lysing the cells in the presence of 4 mM EGTA and 50 mM Tris base and 100 μM digitonin to obtain minimal fluorescence (R_{min}) and by saturating with excess $CaCl_2$ (1.5 mM) for maximal fluorescence (R_{max}). The $[Ca^{2+}]_i$ was calculated with the equation

$$[Ca^{2+}]_i = k_d \times (R - R_{min})/(R_{max} - R_{min}) \times Sf2/Sb2$$

as described by Grynkiewicz et al. (17). A k_d of 225 nM was used.

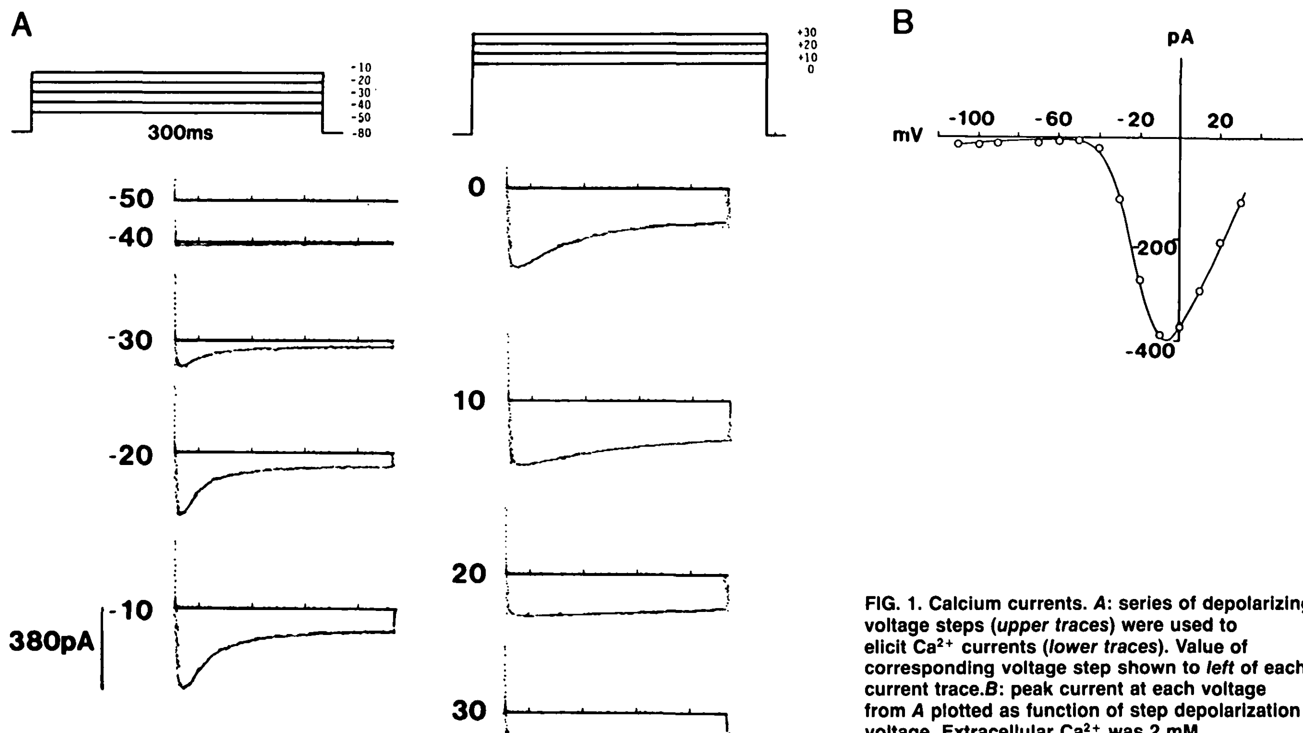

FIG. 1. Calcium currents. *A*: series of depolarizing voltage steps (*upper traces*) were used to elicit Ca^{2+} currents (*lower traces*). Value of corresponding voltage step shown to *left* of each current trace. *B*: peak current at each voltage from *A* plotted as function of step depolarization voltage. Extracellular Ca^{2+} was 2 mM.

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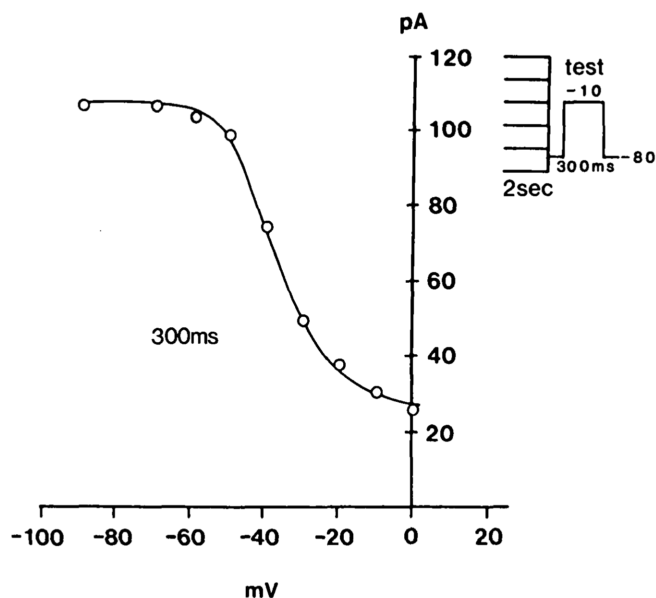

FIG. 2. Channel-availability curve. Voltage protocol was used to examine Ca^{2+} current at −10 mV (*upper right*). Prepulse voltage is plotted on abscissa and current response on ordinate. Curve was computer fitted with relationship $I_{max} = [1 + \exp(V - V_{1/2})/k]^{-1}$, where V is prepulse, $V_{1/2}$ is prepulse giving half-maximal test current (I_{max}), and k is slope factor. $V_{1/2} = -35$ mV; $k = 10$.

RESULTS

Ca^{2+} and Ba^{2+} currents. Ca^{2+} currents elicited from a holding potential of −80 mV in response to depolarizing steps in 10-mV increments up to +30 mV are shown in Fig. 1*A*. Similar results were obtained in 139 cells. The current first appeared between −50 and −40 mV. The current-voltage curve shows that the peak current occurred between −10 and 0 mV (Fig. 1*B*). A rapidly decaying (transient) current can clearly be seen at membrane potentials of −30 to 10 mV. A slowly decaying component is evident over the range −30 to 30 mV. To test the dependence of the current on the values of membrane potential before depolarization, current-availability curves were obtained in 2 mM Ca^{2+}. The membrane potential was held at voltages between −80 and 0 mV for 2 s before applying a test step to −10 mV. The transient component elicited at −10 mV was maximal when the prepulse voltage was more negative than −50 mV. It declined over a voltage range between −60 and −15 mV (Fig. 2). Half of the channels responsible for the transient component were inactivated after a 2-s prepulse to −35 mV. All current was blocked by 0.1 mM Cd^{2+}. Occasionally, an additional small transient component was seen that activated at −60 mV and was completely inactivated at a potential of −50 mV. It resembled the low-threshold current described by Carbone and Lux (11); however, it was not characterized in our studies because of its low frequency of occurrence (1 of ~25 cells). When it did occur, its contribution to the total peak Ca^{2+} current was <10%.

The Ca^{2+} channel also conducts Ba^{2+}. When the Ca^{2+} channel carried Ba^{2+}, the currents had less tendency to decline or run down with time compared with the Ca^{2+} current. Thus, Ba^{2+} is useful as a substitute for Ca^{2+} in experiments in which inhibitory effects of a substance on the Ca^{2+} channel are being examined; in some experiments, Ba^{2+} replaced Ca^{2+} in the external solution. The transient component of the current decayed slower in Ba^{2+} than Ca^{2+} (Fig. 3*A*). When the membrane potential was held at −80 mV and depolarizing pulses were applied, the current initially appeared at −60 to −50 mV. The current-voltage relationship for this cell is shown as the control curve in Fig. 3*C*. Eighty other cells showed equivalent current-voltage relationships. The peak current occurred at approximately −20 mV. The shift to the left in a current-voltage relationship in Ba^{2+} compared with Ca^{2+} has been shown previously (18).

Effects of nimodipine on Ca^{2+} current. In a 2-mM Ba^{2+} bathing solution, nimodipine was applied from a pipette ~10 μm from the cell. At a test voltage of −10 mV, 10^{-7} M nimodipine blocked the peak current by ~85% (Fig. 3*B*). After application of nimodipine, partial current recovery occurred within 60 s. This recovery confirms that the decrease in current observed was due to blockade by nimodipine and not to current run down. An example of the current-voltage relationship obtained in one cell before, during, and after nimodipine is shown in Fig. 3*C*.

Effects of extracellular K^+ on membrane potential. Increases in extracellular K^+ from 5.4 to 40 mM were used to depolarize the HIT cells to initiate Ca^{2+} influx and insulin secretion. Therefore, the effects of 40 mM K^+ on membrane potential were determined for comparison with Ca^{2+} currents elicited at these membrane potentials. Figure 4 shows that the maximum hyperpolarized potential that occurred between bursts of spike activity was reduced from approximately −50 to −35 mV. The action potentials continued at

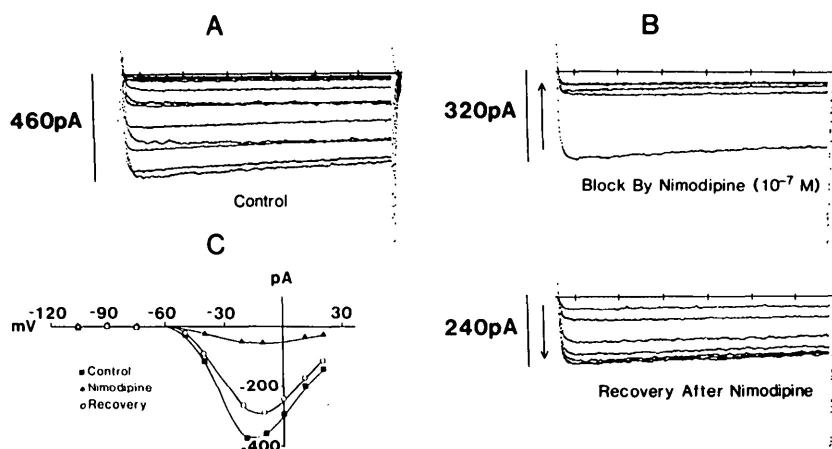

FIG. 3. Ba^{2+} currents. *A*: currents elicited by depolarizing steps from holding potential of −80 mV in 10-mV increments to +30 mV. Extracellular Ba^{2+} was 2 mM. *B*, *Upper panel*: from a holding potential of −80 mV, step to −20 mV was delivered every 5 s. Nimodipine (10^{-7} M) was then applied from pipette placed near cell. Response in 1st test pulse during drug application was reduced (↑). Further reduction occurred over next 2 test pulses. *Lower panel*: recovery after nimodipine pipette is removed from bath (↓). *C*: current-voltage relationship before (■), during (△), and after (○) application of nimodipine to cell in *A* and *B*. Similar results were obtained in 20 cells tested.

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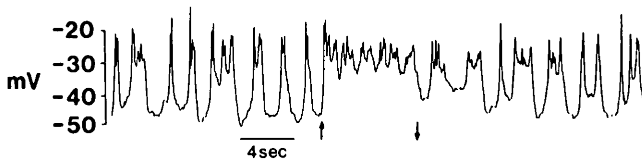

FIG. 4. This intracellular voltage recording illustrates depolarizing effect on spontaneous activity of HIT cell when extracellular K^+ of bath solution is transiently raised from 5.4 to 40 mM (↑).

the peak of the depolarizing phase of the spontaneous activity but were severely reduced in amplitude.

Effects of nimodipine on $[Ca^{2+}]_i$ and insulin secretion. Figure 5*A* illustrates the changes in insulin in the medium when the K^+ concentration was increased from 7 to 40 mM with 2.5 mM Ca^{2+} in the buffer. The insulin content increased from a basal level of 14 to 126 μU/well during the 10-min stimulation period. Nimodipine (10^{-9} M) inhibited 35% of the increase in insulin secretion triggered by depolarization of the cells with K^+. Figure 5*B* illustrates the change in $[Ca^{2+}]_i$ in response to K^+ depolarization in the presence and absence of 10^{-8} M nimodipine. The data from all experiments are summarized in Table 1.

K^+-stimulated insulin secretion, the increase in $[Ca^{2+}]_i$, and the Ca^{2+} currents were inhibited in a concentration-dependent manner (Fig. 6). Maximal inhibition of K^+-stimulated insulin secretion occurred at 10^{-7} M. The concentration giving the IC_{50} was 1.5×10^{-9} M. The IC_{50} for the inhibition of the increase in $[Ca^{2+}]_i$ produced by K^+ depolarization was 6×10^{-9} M. The concentration-response curve in Fig. 6 (*right*) shows the relationship between nimodipine concentration and the percent inhibition of the Ca^{2+} current. The points plotted are averaged values. The IC_{50} for the concentration-response curve was 15×10^{-9} M; maximal effects were reached at 10^{-6} M. Complete inhibition was not observed under the protocol used in these studies.

DISCUSSION

In the last few years, studies directly measuring $[Ca^{2+}]_i$ have shown that Ca^{2+} is the primary second messenger that links a secretagogue stimulus to insulin release. These observations have suggested that voltage-dependent Ca^{2+} channels control $[Ca^{2+}]_i$ and regulate insulin release. Although many laboratories have suggested that Ca^{2+} currents are activated with insulin secretion from isolated islet cells, associated changes in $[Ca^{2+}]_i$ have only been implied. In this study, we have characterized the voltage-gated Ca^{2+} current in an SV40-transformed insulin-secreting β-cell line (HIT cells). Furthermore, by using a combination of electrophysiological and biochemical techniques, we have provided direct evidence linking electrical events at the cell membrane to changes in $[Ca^{2+}]_i$ and insulin secretion.

Voltage-dependent Ca^{2+} channels are present in various excitable cells. The advent of the patch-clamp technique has made it possible to selectively study these channels in small cells with the whole-cell or single-channel recording mode (14). With this technique, various voltage-dependent Ca^{2+} channels have been identified in different tissues, and several Ca^{2+} channels may exist on the same cell membrane. Because several insulin secretagogues, including K^+, amino acids, glucose, and sulfonylureas, induce the opening of voltage-gated Ca^{2+} channels on the β-cell, a more detailed understanding of the gating, conductance, and pharmacological properties of the channel is mandatory for its functional classification.

In cardiac tissue and sensory neurons, where the channel has been most extensively studied, two subtypes have been identified: a T-channel, which has a low threshold of activation and is rapidly inactivated, and an L-channel, with a higher threshold of activation and a longer-lasting inward current (10,11). The T-channel is unavailable for activation if the resting membrane potential is more depolarized than −50 mV. In sensory neurons, a type called the N-channel, with properties intermediate to T- and L-type channels, has also been demonstrated (12). Using HIT cells as a β-cell model, we demonstrated that the range of activation of the Ca^{2+} current is similar to that of the L-channel. It is 90% available for activation at potentials of −50 mV. Studies in other preparations have shown that L- but not T- or N-channels are influenced by 1,4-dihydropyridine. Using nimodipine as a representative of this class of drugs, we have shown that the Ca^{2+} current in HIT cells is almost completely

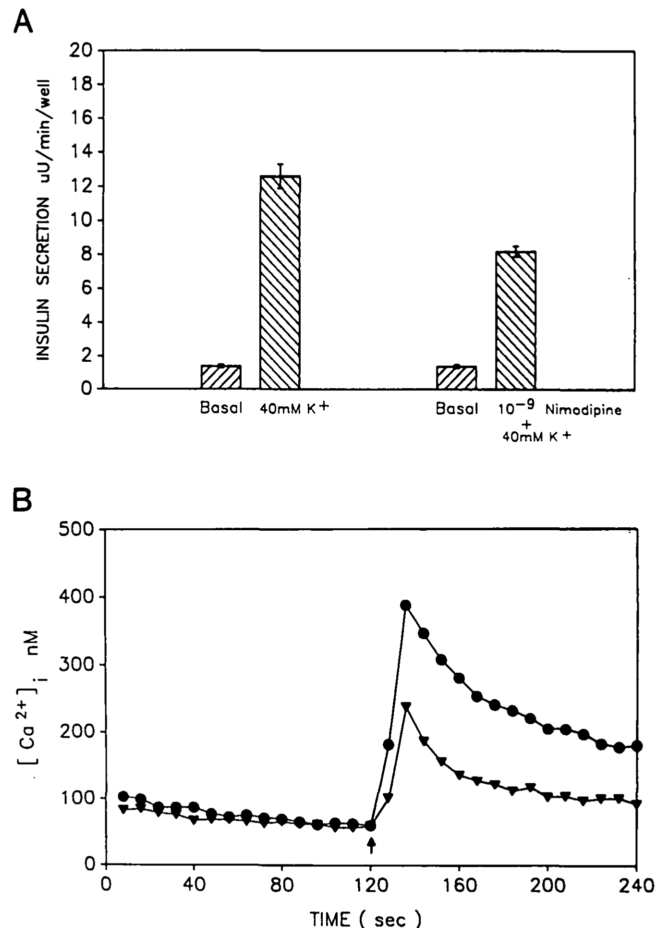

FIG. 5. *A*: Effect of nimodipine on insulin secretion in K^+-stimulated HIT cells. Static incubations of HIT cells were performed to measure insulin release. *Left*, effect of 40 mM K^+ compared to basal insulin secretion. *Right*, effect of 10^{-9} M nimodipine on K^+-stimulated secretion. *B*: effect of nimodipine on $[Ca^{2+}]_i$ in K^+-stimulated HIT cells. $[Ca^{2+}]_i$ levels were measured in suspended HIT cells loaded with Fura 2. ●, Effect of 40 mM K^+ on $[Ca^{2+}]_i$; ▼, $[Ca^{2+}]_i$ when cells were treated with 10^{-8} M nimodipine before addition of K^+.

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TABLE 1
Changes in insulin secretion and free cytosolic calcium in HIT cells

Pretreatment	Insulin secretion (μU · min⁻¹ · well⁻¹) Insulin levels: Basal	Stimulated	*n*	*P*	Free cytosolic Ca^{2+} (nM) Ca^{2+} levels: Basal	Stimulated	*n*	*P*
	1.4 ± 0.1	12.6 ± 0.7	12	<.001	61 ± 4	381 ± 15	12	<.001
10^{-10} M nimodipine	1.8 ± 0.2	12.4 ± 0.6	12	<.001	74 ± 15	370 ± 40	5	<.001
10^{-9} M nimodipine	1.4 ± 0.1	8.2 ± 0.3	12	<.001	79 ± 14	347 ± 25	5	<.001
10^{-8} M nimodipine	1.5 ± 0.2	5.0 ± 0.3	12	<.001	57 ± 6	225 ± 17	3	<.002
10^{-7} M nimodipine	1.3 ± 0.1	2.0 ± 0.3	12	<.002	63 ± 3	141 ± 6	4	<.001
10^{-6} M nimodipine	1.1 ± 0.1	1.5 ± 0.2	12	<.002	58 ± 7	91 ± 9	2	<.02

Values are means ± SE. Stimulating agent, 40 mM K^+. *n* = number of determinations. *P* values are vs. basal levels.

inhibited by dihydropyridines. Because a component of the antagonistic action of dihydropyridine is voltage dependent (19), with preference of action on the inactivated state of the channel, complete blockage of the Ca^{2+} current would be expected only if the membrane potential could be held in a sufficiently depolarized state (channel inactivated) for long enough periods to produce complete inactivation of the channels. Furthermore, previous studies with this class of drugs in ventricular cells have shown that they may have dual effects, stimulatory or inhibitory, depending on the membrane potential (20).

A partial agonist effect of nimodipine can also explain the inability to achieve complete inhibition of the inward Ca^{2+} current in β-cells. Note that the small current remaining at the higher concentrations of nimopidine did not show kinetics of the T- or N-channels; it retained the characteristics of the L-channel. These properties of the Ca^{2+} channel are therefore consistent with the notion that a single class of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels exists on β-cell plasma membranes that is similar to the L-type or high-threshold channel identified on other excitable cells. The results with nimodipine and the inactivation range of the current do not support the recent suggestion that HIT cells contain two types of Ca^{2+} channels (13). Currents in other β-cell types may be compared with the HIT cells. The currents recorded by Satin and Cook (6), with Ba^{2+} as the charge carrier in neonatal islet cells, were similar to those recorded in our study. The Ca^{2+} currents measured by Rorsman and Trube (8) in pancreatic islets of NMRI mice also showed similar kinetics to the HIT cells. Findlay and Dunne (7) recorded Ca^{2+} currents in the RINm5F cell line. Although the currents were not separated from K^+ currents, the RINm5F cells appeared to have a more rapid transient component. A recent report of single Ca^{2+} channels in RINm5F cells supports the observation that L-channels are responsible for currents recorded in Ba^{2+} (21).

Although the primary insulin secretagogues glucose, amino acids, and sulfonylureas induce cell depolarization leading to opening of the voltage-dependent Ca^{2+} channels, there is no evidence indicating a direct effect of these substances on the Ca^{2+} channel. This suggests an alternate initial site of interaction for these secretagogues with the β-cell. There is mounting electrophysiological evidence that an ATP-sensitive K^+ channel controls the resting membrane potential in β-cells. Several laboratories, including ours, have shown that metabolites of glucose, ATP, and sulfonylureas inactivate this channel, causing decreased K^+ conductance and subsequent cell depolarization (22–24). These observations combined with those of our study suggest that glucose, amino acids, and sulfonylureas initially inactivate ATP-sensitive K^+ channels on the cell membrane, which then leads to cell depolarization and opening of the voltage-gated Ca^{2+} channels.

After opening of the voltage-dependent Ca^{2+} channels, Ca^{2+} moves down the electrochemical gradient into the β-cell, raising $[Ca^{2+}]_i$ and triggering insulin secretion. To cor-

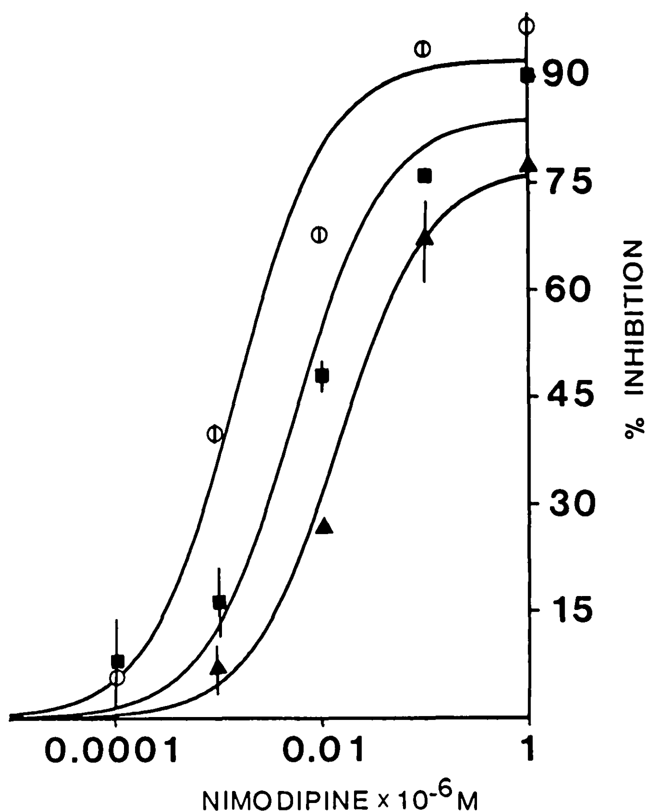

FIG. 6. Concentration-response curve to nimodipine is plotted as percent inhibition of Ca^{2+} current (▲). Data from experiments in which Ca^{2+} and Ba^{2+} were used were pooled (*n* = 20). Concentration-response curves for insulin secretion (○) and $[Ca^{2+}]_i$ (■) in response to depolarization are also shown (*n* = 3–12). Values are means ± SE. Curves are least-squares fits of 1-site model.

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relate the effects on $[Ca^{2+}]_i$ with the electrophysiological changes, we used a 40-mM concentration of K^+ to stimulate the cells. At this concentration, the membrane potential is depolarized to at least −30 to −35 mV, which is in the range of Ca^{2+}-current activation. A comparison of the inhibition by nimodipine of the depolarization-induced Ca^{2+} current and the rise in $[Ca^{2+}]_i$ showed excellent correlation between these two variables with an IC_{50} of 15×10^{-9} and 6×10^{-9} M, respectively. The IC_{50} for the effect on insulin secretion was also close, 1.5×10^{-9} M. In this study, the IC_{50} for the nimodipine inhibition of the K^+-induced effects on the Ca^{2+} current, $[Ca^{2+}]_i$, and insulin secretion ranged just over 1 order of magnitude, showing the close linkage between these successive events in stimulus-secretion coupling in the β-cell. The insulin-secretion studies were performed at 37°C with HIT cell monolayers. However, the Fura 2 measurements were performed with suspended HIT cells at 20°C to minimize dye leakage (2,4). The small difference in IC_{50} can therefore be easily explained by differences in the experimental protocols. We and others have shown that differences may exist depending on whether the k_d of dihydropyridine is determined from electrophysiological or biochemical experiments (25,26). Furthermore, as observed in the electrical measurements, nimodipine did not completely inhibit the rise in $[Ca^{2+}]_i$, consistent with its partial agonistic action. These studies indicate that the increase in β-cell $[Ca^{2+}]_i$ that has been observed on cell depolarization in many laboratories can be accounted for on the basis of the influx of this ion through a single class of voltage-dependent Ca^{2+} channels.

We have previously demonstrated that depolarization of the HIT cells with K^+ specifically phosphorylates three Ca^{2+}-dependent proteins that may be important in exocytosis (27). This phosphorylation, the rise in $[Ca^{2+}]_i$, and insulin secretion can also be blocked by the Ca^{2+}-channel antagonist verapamil.

In summary, this study presents direct evidence linking membrane depolarization with the opening of a single class of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} and the rise in $[Ca^{2+}]_i$ triggers insulin release.

ACKNOWLEDGMENTS

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