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The L-type Voltage-Gated Ca^{2+} Channel Is the Ca^{2+} Sensor Protein of Stimulus−Secretion Coupling in Pancreatic Beta Cells†

Michael Trus,‡ Richard F. Corkey,§ Rafael Nesher,|| Ann-Marie T. Richard,§ Jude T. Deeney,§ Barbara E. Corkey,§ and Daphne Atlas*,‡

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel, Obesity Research Center, Boston University School of Medicine, Boston, Massachusetts 02118, and Endocrinology and Metabolism Service, Department of Medicine, The Hebrew University, Hadassah Medical Center, Jerusalem, Israel

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ABSTRACT: L-type voltage-gated Ca^{2+} channels (Cav1.2) mediate a major part of insulin secretion from pancreatic β-cells. Cav1.2, like other voltage-gated Ca^{2+} channels, is functionally and physically coupled to synaptic proteins. The tight temporal coupling between channel activation and secretion leads to the prediction that rearrangements within the channel can be directly transmitted to the synaptic proteins, subsequently triggering release. La^{3+}, which binds to the polyglutamate motif (EEEE) comprising the selectivity filter, is excluded from entry into the cells and has been previously shown to support depolarization-evoked catecholamine release from chromaffin and PC12 cells. Hence, voltage-dependent trigger of release relies on Ca^{2+} ions bound at the EEEE motif and not on cytosolic Ca^{2+} elevation. We show that glucose-induced insulin release in rat pancreatic islets and ATP release in INS-1E cells are supported by La^{3+} in nominally Ca^{2+}-free solution. The release is inhibited by nifedipine. Fura 2 imaging of dispersed islet cells exposed to high glucose and La^{3+} in Ca^{2+}-free solution detected no change in fluorescence; thus, La^{3+} is excluded from entry, and Ca^{2+} is not significantly released from intracellular stores. La^{3+} by interacting extracellularlly with the EEEE motif is sufficient to support glucose-induced insulin secretion. Voltage-driven conformational changes that engage the ion/EEEE interface are relayed to the exocytotic machinery prior to ion influx, allowing for a fast and tightly regulated process of release. These results confirm that the Ca^{2+} channel is a constituent of the exocytotic complex [Wiser et al. (1999) *PNAS 96,* 248−253] and the putative Ca^{2+}-sensor protein of release.

It is widely accepted that in pancreatic β-cell the intracellular Ca^{2+} rise generated by Cav1.2 activation is responsible for vesicle fusion and insulin release. In this nearly axiomatic model, membrane depolarization initiates Ca^{2+} entry and binding to the vesicular protein synaptotagmin, which together with the soluble *N*-ethylmaleimide attachment protein receptor (SNARE) complex proteins ultimately mediates vesicle fusion and exocytosis (*1*−*3*).

Integral to this scheme is that Ca^{2+} entry via the voltage-gated calcium channels (VGCC) is a necessary part of the exocytotic process where the channel acts to regulate Ca^{2+} entry and subsequent secretion.

We proposed an alternative model of stimulus−secretion coupling based on a wealth of biochemical and electrophysiological data (*4*−*12*). We demonstrated a highly specific physical (*12*−*14*) and functional link of the cytoplasmic domains between repeats II and III of the α_1 subunit of VGCC with the synaptic proteins syntaxin 1A, SNAP-25, and synaptotagmin (*4*, *5*, *15*). When injected into single insulin-secreting mouse β-cells, the cytoplasmic II−III domain ($Lc_{753-893}$) of the $\alpha_1$1.2 subunit of Cav1.2 inhibited the transient rise in capacitance in response to channel opening. This suggested that $Lc_{753-893}$ competition with the endogenous Cav1.2 for synaptic proteins disrupted the spatial coupling of Cav1.2 with the secretory apparatus (*6*, *8*, *9*, *16*). A detailed analysis revealed the loss of the rapid component of insulin secretion in mouse β cells injected with $Lc_{753-893}$ (*17*). These results were further confirmed by capacitance measurements in the β Cav1.2 (−/−) mouse in which a beta cell-selective ablation of the gene encoding the L-type Ca(v)1.2 channel resulted in loss of the fast component of secretion (*18*). Similar inhibition of evoked release by the II−III cytosolic peptide (synprint) has been reported in superior cervical ganglion neurons (*19*).

Our proposed model suggests that the channel operates as a Ca^{2+} sensor protein of secretion, upstream to Ca^{2+} entry into the cell (*6*, *8*, *9*, *15*, *20*−22). The tight coupling of the channel with the synaptic proteins would facilitate propagation of conformational changes induced during channel opening, to syntaxin 1A, SNAP-25, and synaptotagmin to trigger secretion (*8*, *9*). A change in the channel conformation would be enough to trigger the fusion and subsequent release of a pool of docked and primed vesicles coupled to the VGCC ('channel associated vesicles' (*8*, *9*).

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* To whom correspondence should be addressed. Phone: 972-2-6585406. Fax: 972-2-6585413. E-mail: datlas@vms.huji.ac.il.

‡ The Hebrew University of Jerusalem.

§ Boston University School of Medicine.

|| The Hebrew University, Hadassah Medical Center.

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The Ca^{2+}-channel pore contains a Ca^{2+} binding site consisting of four negatively charged glutamate residues, the EEEE locus. The glutamate residues are highly conserved throughout the VGCC family and thought to underlie the basis of VGCC selectivity (*23*, *24*). The EEEE locus binds Ca^{2+} with high affinity in the micromolar range. During depolarization the high-affinity site transforms into a low-affinity site that binds Ca^{2+} in the millimolar range, and the channel then becomes permeant to generate ion currents (*23*−*26*). In our model, the voltage-driven transitions from high to low affinity at the selectivity filter, prior to ion permeation, is an essential step for triggering secretion.

When the permeable Ba^{2+} or Sr^{2+} were substituted for Ca^{2+}, the kinetics of the calcium channel coupled to syntaxin were further modified (*7*). These results indicated a direct link between pore occupancy and the channel coupling to the synaptic proteins, suggesting a regulatory site for secretion at the pore, upstream to Ca^{2+} entry. To probe voltage-driven rearrangements within an ion occupied-selectivity filter, a cation was sought that could bind to the channel cavity without entering the cell and yet mediate release. We found that La^{3+}, an impermeable cation, with an ionic radius (1.061 Å) similar to Ca^{2+} (0.99 Å), could mediate stimulus−secretion coupling via Cav1.2 in PC 12 and chromaffin cells (*20*). Trivalent (Gd^{3+} and Eu^{3+}) and divalent cations (Cd^{2+}) with smaller ionic radius however did not support release, indicating that the EEEE locus constitutes a size-discriminatory binding site at the channel cavity.

In the current study we examined the ability of La^{3+} to substitute for Ca^{2+} in supporting glucose-induced insulin release in rat pancreatic islets. Our results are consistent with the proposed model in which the contact of La^{3+} at the selectivity filter during membrane depolarization is sufficient to support release, possibly by conferring a release-competent configuration on the channel.

The results broaden the basis of our heuristic model of the calcium channel as the primary Ca^{2+}-sensor protein in a variety of secretory systems, involving either neurotransmitter or hormone release, where activation of exocytosis is mediated by the L-type voltage-gated calcium channel.

MATERIALS AND METHODS

INS-1E cells were a generous gift from C. Wollheim (*27*). Collagenase was from Worthington, USA. Purified bovine albumin was from AMRESCO (Cleveland, OH). An insulin radioimmunoassay kit was obtained from Linco (Millipore, St. Charles, MO). The Enliten ATP assay kit was from Promega (Madison, WI). Cell culture media components were from Gibco-Invitrogen (CA, USA). Other general reagents were from Sigma-Aldrich (St. Louis, MO), Merck (New Jersey), or Frutarom (Haifa, Israel).

Solutions for Incubations. The Krebs solution used for batch incubations of INS-1E cells or intact islets consisted of 140 mM NaCl, 4.5 mM KCl, 0.4 mM KH_2PO_4, 0.15 mM $NaHPO_4$, 2.5 mM $NaHCO_3$, 0.5 mM $MgSO_4$, 20 mM Hepes, pH 7.4, 0.05% albumin, plus addition of $CaCl_2$, $LaCl_3$, and glucose as shown in the figures and tables. The bicarbonate ion was reduced to prevent formation of insoluble lanthanum carbonate, and the primary buffer was HEPES. Stock solutions of 100 mM $CaCl_2$ or 100 mM $LaCl_3$ were used for preparing the Krebs solution. The $LaCl_3$ solution was always prepared fresh immediately prior to adding to the Krebs solution to limit formation of precipitates.

ATP Release in INS-1E Cells. INS-1E cells were grown as previously described (*27*). Prior to measurement of secretion the cells were seeded in a 96-well plate and grown for 2 days to a semi-confluent state. For batch incubations of INS-1E cells, medium was removed and the cells were washed twice with 200 μL of Krebs medium without $CaCl_2$. Then the cells were pretreated with 200 μL of medium containing Krebs. After medium removal 70 μL of Krebs containing low glucose (3 mM) with either 100 μM $LaCl_3$ or 1.0 mM $CaCl_2$ were added. After 30 min incubation at 37 °C the medium was collected and saved for ATP assay. An additional 70 μL of medium containing 20 mM glucose was added. At the end of the 30 min incubation this medium was collected and assayed for ATP content, measuring luminescence in a 96-well plate. Numbers in the figures were expressed as arbitrary luminescence units or percent basal release. However, the luciferase assay was ascertained to give similar values of luminescence when the ATP standards were prepared in Krebs medium assayed containing either high or low glucose and either Ca^{2+} or La^{3+}.

Intact Rat Islets. Islets were isolated from rat pancreas by collagenease digestion followed by centrifugation through Histoplaque (Sigma, USA; (*28*)) and subsequent hand picking of islets. Before use the islets were routinely incubated overnight at 37 °C in RPMI 1630 medium containing 10% Fetal Calf Serum and Penicillin plus Streptomycin.

For the perifusions, 60 islets per chamber were loaded and the flow rate was 0.3 mL/min. Samples were collected at 1 min intervals for insulin determination by radioimmunoassay.

The general perifusion protocol was as follows: A 30 min perifusion with normal Ca^{2+}-containing Krebs was followed by a 30 min equilibration with Krebs containing neither Ca^{2+} nor La^{3+}. Then, the islets were perifused for 30 min with Krebs containing low glucose and either 2.0 mM Ca^{2+} or 0.1 mM La^{3+}. Sample collection began in the last 10 min of this perifusion. Finally, the islets were stimulated with high glucose (20 mM) in the presence of either Ca^{2+} (2.0 mM) or La^{3+} (0.1 mM) and 1 min fractions collected for 40 min.

Batch Incubations of Islets. Islets were equally distributed into 1.5 mL Eppendorf centrifuge tubes. Five to eight islets per tube were used for each experiment. Between all washes the islets were centrifuged briefly for ∼5 s at 1000 rpm before aspirating the medium. Initially, the islets were washed 2 times with 1.0 mL of Krebs without Ca^{2+} or La^{3+}. Then the islets were preincubated 30 min with 250 μL of Krebs containing either 2.0 mM $CaCl_2$ or 0.1 mM $LaCl_3$ at 37 °C. For experiments with nifedipine the islets were pretreated with 250 μL of Krebs containing 5 μM nifedipine for 10 min in the absence of either Ca^{2+} or La^{3+} before pretreatment with Krebs containing nifedipine plus Ca^{2+} or La^{3+} as indicated above.

After pretreatment the medium was aspirated, 250 μL of the same medium was added to each tube, and the tubes were incubated at 37 °C for 30 min. Then 230 μL of each tube was saved for insulin assay. Glucose stimulation begun by adding 250 μL of Krebs containing 20 mM glucose, and the islets were incubated at time periods as indicated at 37 °C. Then, 230 μL was collected from each tube and stored at −20 °C for insulin assay.

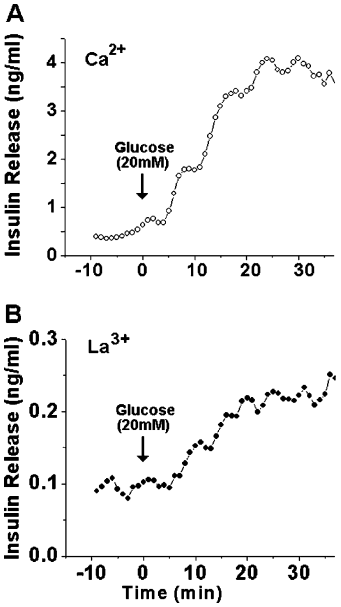

FIGURE 1: Similar onset of glucose evoked insulin release in pancreatic beta cells using Ca^{2+} or La^{3+} in nominally Ca^{2+}-free solution. Insulin secretion in intact rat islets was carried out by perifusion with (A) 2 mM Ca^{2+} or (B) 0.1 mM La^{3+}. The preperifusion protocol and preliminary wash out of islets is described in the Experimental Procedures. Each perifusion consisted of 60 islets per chamber. The flow rate was 0.3 mL/min. One minute samples were collected 10 min prior to stimulation and for 40 min thereafter. Each experiment was repeated three times using different preparations of pancreatic islets. Each point is the average of three consecutive time points.

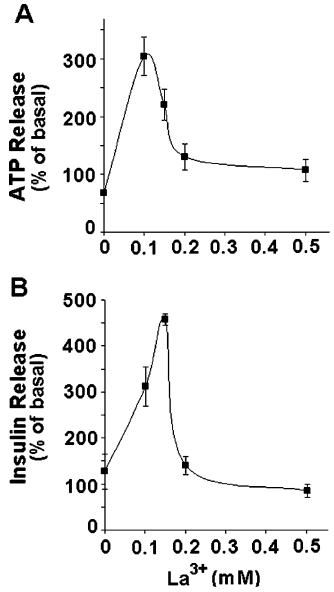

FIGURE 2: Concentration dependence of glucose-induced insulin and ATP release in INS-1E and pancreatic cells exposed to La^{3+} in nominally Ca^{2+}-free solution. ATP release was monitored in INS-1E cells and insulin release in pancreatic rat islets with increasing La^{3+} concentrations. (A) INS-1E insulinoma cells were incubated in batches at 2 mM glucose and different levels of La^{3+} as indicated in the graph. After 30 min incubation at 37 °C the buffer was removed for ATP assay. Then 20 mM glucose and the appropriate level of La^{3+} were added for an additional 30 min incubation period. Values are expressed as percent of ATP release induced by 20 mM glucose compared to 2 mM glucose ($G_{20}/G_2 \times 100$). (B) Using 4−6 wells for each La^{3+} concentration, five intact rat islets were incubated at various La^{3+} levels with 2 mM glucose in Krebs medium. After 30 min the media were removed and saved for insulin assay. Media were then replaced with high glucose (20 mM) containing Krebs buffer. After 30 min media were collected for insulin assay. Values are expressed as a percent stimulation by G_{20} compared to the basal glucose release ($G_{20}/G_2 \times 100$) and represent data from two independent experiments.

Fura 2 Measurements. Islet cells were dispersed by trypsin treatment of islets and grown on cover slips for 2−4 days. The dispersed cells were incubated with 3 μM Fura 2-AM for 1 h in Krebs containing low glucose (2 mM). The Fura containing Krebs was removed, and the cells were washed twice with 1.5 mL of Krebs lacking Ca^{2+} or La^{3+}. Then the cells were incubated with 1 mL of Krebs containing 2 mM glucose and either 2 mM $CaCl_2$ or 0.1 mM $LaCl_3$ for the initial measurements. To begin the experiment, 0.5 mL of Krebs containing 60 mM glucose was added to the Krebs already in the dish to achieve a final glucose level of 20 mM.

RESULTS

High Glucose Induces Insulin Secretion in Pancreatic β Cell Exposed to La^{3+} in Ca^{2+} Free Solution. We perifused rat islets in the presence of either Ca^{2+} or La^{3+} and measured the time course of insulin release (Figure 1A,B). Pancreatic β cells perifused with 20 mM glucose elicited insulin secretion in the presence of 2 mM Ca^{2+}, showing a 9-fold insulin release over basal release observed in low glucose (2 mM). When 0.1 mM La^{3+} was substituted for Ca^{2+}, 20 mM glucose induced a 2.5-fold increase in insulin release over basal (Figure 1B). Comparing La^{3+} to Ca^{2+}, the degree of high glucose stimulated insulin release in perifused β-cells correlated proportionately with ATP release during batch incubations of insulinoma cells (INS 1E) (data not shown). Thus, both assays show similar lower but significant efficacy of La^{3+} at supporting glucose-induced secretion.

Glucose-induced insulin release exhibited a similar time course in the presence of either Ca^{2+} or La^{3+} parallel to previous observations of the time course of dopamine release in depolarized chromaffin cells when Ca^{2+} was compared to La^{3+} (*20*).

Concentration Dependence of Insulin and ATP Release Supported by La^{3+}. Our previous work with PC12 and bovine chromaffin cells indicated that La^{3+} at an optimal level of 0.1−0.2 mM supported depolarization-evoked release of catecholamines in nominally Ca^{2+}-free solution (*20*). We used the conditions in these previous experiments as a guide for our study of the pancreatic β cell and INS-1E. Since ATP is co-released with insulin in secretory granules, our preliminary studies used ATP release as a marker of hormone release from batch incubated INS-1E cells. High glucose (20 mM) was able to stimulate ATP release by ~8-fold over basal release in the presence of 2 mM Ca^{2+}, and when 2 mM Ca^{2+} was replaced by 0.1 mM La^{3+}, high glucose stimulated ATP release 3-fold (data not shown). Less effective release by La^{3+} is in agreement with lower efficiency of release, previously shown when Ba^{2+} or Sr^{2+} substituted for Ca^{2+} (*29*).

Next we determined ATP release in INS-1E cells and insulin secretion in batch incubated intact rat pancreatic islets at increasing La^{3+} concentrations, as indicated (Figure 2A,B). Glucose stimulation of ATP release monitored from INS-1E cells showed a narrow range of concentration dependency on La^{3+} (Figure 2A) similar to glucose-induced insulin

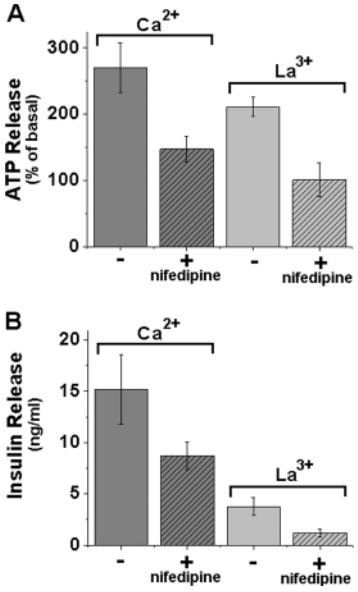

FIGURE 3: Nifedipine inhibits glucose-induced secretion of insulin and ATP in INS-1E and pancreatic beta cells exposed to La^{3+} in nominally Ca^{2+}-free solution. (A) INS-1E cells were incubated for 30 min in Krebs containing 2 mM glucose. The media (G_2) were collected for ATP assay and then replaced with media containing 20 mM glucose for an additional 30 min incubation, and the media (G_{20}) were collected for ATP assay. Release is expressed as a percentage of basal release in each of the four wells per condition. Incubations were in 1 mM Ca^{2+} or 0.1 mM La^{3+} with (hatched) or without (solid) 5 μM nifedipine. (B) Rat intact islets were incubated, eight islets per tube, for 30 min at 2 mM glucose followed by 30 min with 20 mM glucose. Insulin release was assayed in the medium from G_2 and G_{20} aliquots. The results are expressed as fold insulin release due to the switch to high glucose G_{20}/G_2. Incubations were carried out in 2 mM Ca^{2+} or 0.1 mM La^{3+} with (hatched) or without (solid) 5 μM nifedipine. Each condition of release was done in quadruplicate.

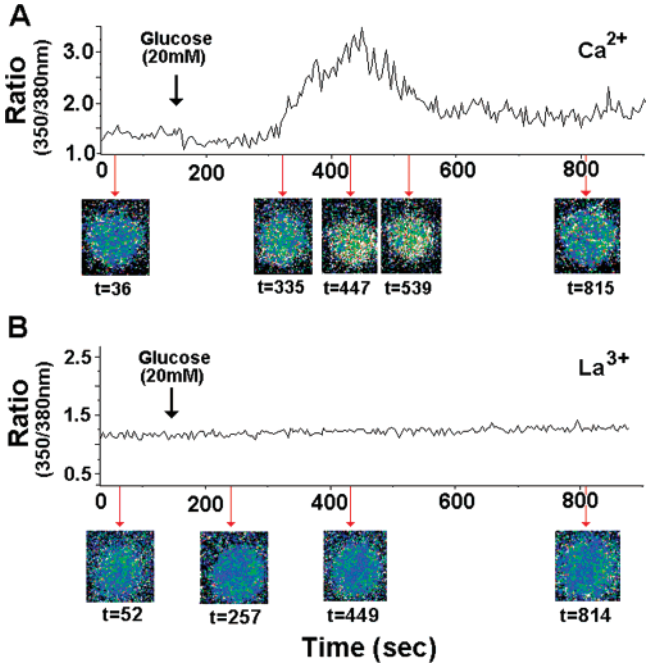

FIGURE 4: Fura 2 imaging of dispersed islets cells exposed to high glucose and La^{3+} in Ca^{2+}-free solution detects no change in fluorescence. Trypsin-dispersed rat islet cells were loaded with Fura 2 AM and then imaged with 2 mM glucose in the presence of 2 mM Ca^{2+} (A) or 0.1 mM La^{3+} (B). Glucose was added where indicated to a final concentration of 20 mM. These experiments were carried out with several preparations of trypsin-dispersed islets. Representative images with their respective time in seconds during the trace are shown below each trace. Fluorescence was detected in 42 out of 53 cells when Ca^{2+} was used, while none of the 38 cells tested gave fluorescence when La^{3+} substituted for Ca^{2+} in the extracellular medium.

release in rat islets (Figure 2B). The dose−response curves showed a maximal release at 0.1−0.2 mM La^{3+} and a sharp decrease at higher concentrations (Figure 2B) similar to La^{3+} dependency in adrenal cells (*20*). It should be noted that at concentration above 0.2 mM the solubility of lanthanum decreases due to formation of insoluble lanthanum carbonate and could explain the drastic reduction in ATP and insulin secretion.

Nifedipine Inhibits Secretion of Insulin and ATP in Pancreatic Beta Cells Exposed to La^{3+}. Since there are multiple targets for La^{3+} binding as well as nonspecific effects of La^{3+} on cells, we determined whether the glucose stimulated insulin release with La^{3+} in nominally Ca^{2+}-free solution was mediated through binding at the L-type Ca^{2+} channel. For that we used the allosteric and selective blocker of the L-type channel, nifedipine (*30*−*32*). Ca^{2+} binding at the pore is allosterically coupled to dihydropyridine (DHP) binding (*33*). Hence, if nifedipine affects both Ca^{2+} and La^{3+} binding at the pore, then nifedipine inhibition of secretion in Ca^{2+} and in La^{3+} would strongly indicate that both cations were acting through a common site. In batch incubated INS-1E cells, ATP release in a Ca^{2+} solution was inhibited by ~50% by nifedipine. In La^{3+}-containing solution, the ATP release in INS-1E cells was ~60% inhibited (Figure 3A). In batch incubated rat pancreatic islets, with Ca^{2+}, nifedipine reduced glucose-induced insulin release by ~50% (Figure 3B). Similarly, insulin secretion in La^{3+} was inhibited by 70%. These results show that La^{3+} support of glucose-stimulated secretion like Ca^{2+} was largely mediated by binding to the L-type calcium channels, as both are similarly affected by DHP binding at a selective site of this channel.

La^{3+} Is Excluded From Entry Into the Pancreatic Beta Cells. Pancreatic islets were prelabeled with 3 μM Fura 2-AM, washed, and incubated with low glucose (2 mM) in a solution containing 2 mM Ca^{2+}. Elevating glucose to 20 mM resulted in the expected increase in Fura2 fluorescence (ratio of 350/380 nm) in 42 of 53 cells tested (Figure 4A). In contrast, elevating glucose to 20 mM in a solution containing 100 μM La^{3+} substituted for 2 mM Ca^{2+} failed to evoke response in any of the 38 cells measured (Figure 4B). Since exclusion of La^{3+} from entry into the cells was the main issue of the present study, this result clearly shows at the single-cell level that the basal fluorescence ratio of cells incubated in La^{3+} solution remains constant throughout the experiment. Previously it was demonstrated that in permeabilized adrenal cells the EC_{50} for La^{3+} was shifted to the left of that for Ca^{2+} by 1 order of magnitude (*34*). Therefore, the sensitivity of the Fura2 to La^{3+} with a detection limit of 1 pM (*20*, *35*) indicates that La^{3+} entry is clearly below the required concentration for secretion.

DISCUSSION

Secretion in pancreatic islets, similar to secretion in chromaffin, pheochromocytoma (PC12), and retinal cells, is largely mediated by activation of Cav1.2, the L-type voltage-gated Ca^{2+} channel. Other channels, e.g., N- (Cav2.2), P/Q-

(Cav2.1), R-(Cav2.3), or T-(Cav3.1)-type are also responsible for secretion in these cells but to a smaller extent (*36*−*38*).

A linkage between secretion and ion bound at the selectivity filter of Cav1.2 has been recently demonstrated, both biochemically and amperometrically, in bovine adrenal chromaffin and rat PC12 cells (*20*). Ion binding at the channel cavity was dissociated from ion influx by substituting the impermeable La^{3+} for Ca^{2+} (*20*). At concentrations of 100−200 μM and in the absence of Ca^{2+}, La^{3+} supported depolarization evoked release without entering the cells establishing a signaling role for Cav1.2 in secretion, upstream to ion entry (*20*). La^{3+} was used in the absence of Ca^{2+} because in its presence it competes for the high-affinity site at the pore of the channel and acts like a channel blocker at submicromolar concentrations (*39*). Since La^{3+} and to a lesser extent Ce^{3+} and Pr^{3+} supported evoked secretion without entry into the cell, the VGCC was proposed to be the Ca^{2+} sensor for secretion (*7*−*9*, *20*).

Here we tested this concept in the pancreatic β cell, which has a vastly different mechanism of secretion from the chromaffin cell. In islets the pathway of glucose-induced insulin secretion involves both glucose metabolism and oxidative phosphorylation (*40*), and yet a final common event in both organs involves the VGCC, in particular the Cav1.2.

Glucose Promotes Insulin Secretion with La^{3+} in Nominally Ca^{2+}-Free Solution. Rat pancreatic islets stimulated with glucose in the presence of La^{3+} in a nominally Ca^{2+}-free solution elicited insulin secretion at a slower rate than observed in islets perifused with Ca^{2+}. This is analogous to an agonist that activates its corresponding receptor less efficiently. The slower rate of secretion and thus the lower total secretion in La^{3+} are thus predictable. The differences in Ca^{2+}, Sr^{2+}, Ba^{2+}, or La^{3+} binding to EGTA are comparable to binding differences to the related ion-binding site created by the EEEE locus. Based on interaction differences of the permeating and non-permeating cations at the EEEE locus, the consequent rearrangements within the cavity are likely to lead to differences in release efficacy in adrenal cells (*20*). A poorer performance and lower efficacy of secretion was observed when Sr^{2+} or Ba^{2+} were substituted for Ca^{2+} in rat pancreatic -islet beta cells (*29*). La^{3+}, and to a lesser extent Sr^{2+}, and Ba^{2+}, might generate a less favorable 'releasing' channel conformation, which in turn decreases the efficacy of secretion.

Glucose-induced insulin secretion is mediated by glucose metabolism that causes the closure of the K_{ATP} channel leading to membrane depolarization and opening of voltage-gated Ca^{2+} channels in beta-cells (*41*, *42*). Using batch incubation, La^{3+} was effective at supporting either glucose- or high KCl-evoked ATP release in INS-1E cells. This indicates that La^{3+}-mediated secretion can occur when glucose metabolism is bypassed and the Ca^{2+} channel is directly activated.

It is well accepted that the dominant forces in the conducting pore are binding and repulsion of ions within the selectivity filter (*43*−*47*). The voltage-driven rearrangements affect resident ions interaction at the selectivity filter, in turn affecting channel kinetics. These perturbations can be transmitted directly to the exocytotic protein associated with the channel in the excitosome complex, enabling a rapid signaling means for eliciting secretion (*4*−*9*, *21*, *22*, *48*−*51*). The fast phase of insulin release could be accounted for by the fusion of a readily releasable pool of vesicles termed "channel associated vesicles" which are primed and docked awaiting a signal (*8*, *9*, *17*, *19*). This model of depolarization-induced release of vesicles associated with the channel is consistent with the co-localization of VGCC and vesicles at the release sites (*52*) shown in several elegant capacitance, fluorescent, and TIRFM imaging studies (*17*, *53*−*57*).

The concentration dependency of both the insulin and the ATP release showed a comparable maximum at ∼0.1 mM La^{3+}. Throughout our study, parallel qualitative results were obtained whether ATP release was monitored in the insulinoma cell line or insulin release was assayed in the intact islets.

As seen in adrenal cells, evoked release was inhibited by nifedipine, a selective Cav1 allosteric effector (*33*). The specific inhibition of release by nifedipine demonstrates that, indeed, La^{3+} support of secretion is mediated via binding to Cav1-type channel(s) and not by other pathways. Furthermore, release in response to high glucose in either cation is mediated primarily by Cav1 activation, consistent with the selectivity filter (EEEE locus) being the common site for these ions that lead to secretion.

La^{3+} Does Not Enter Into Pancreatic β Cells and Does Not Increase $[Ca^{2+}]_i$. If La^{3+} was involved in inducing insulin release by elevating $[Ca^{2+}]_i$ from intracellular stores and/or permeating into the cell, we would expect to see changes in the fluorescence ratio of Fura 2 (350/380 nm) like those observed with Ca^{2+}. Since Fura 2 is 10 000-fold more sensitive to La^{3+} than Ca^{2+}, we should have been able to detect La^{3+} concentrations as low as ∼1−3 pM (*20*, *35*). Our present results show that pancreatic islets responded to glucose stimulation in the presence of La^{3+} but detected no change in the fluorescence ratio at 350/380 nm.

Alternatively, it can be argued that entry of La^{3+} at concentrations below the lower limit of the detection system, calculated to be ∼1 pM (10^{-12}), is responsible for secretion. If this argument is right then the Ca^{2+} sensor protein is activated by <1 pM concentrations of La^{3+}. Since in permeabilized adrenal chromaffin cells secretion required >10 μM La^{3+} (*34*), this possibility is rather unlikely. These Fura 2 results are consistent with previous fluorescence studies in bovine chromaffin cells (*20*) and rat chromaffin cells (Ning-Guo, personal communication), where it was shown that La^{3+} neither enters into the cells nor causes Ca^{2+} release from intracellular stores.

CONCLUSIONS

Cav1.2 mediates insulin secretion from rat pancreatic islets and depends on the presence of Ca^{2+} ions in the external solution. Here we demonstrate that the trivalent cation La^{3+}, which binds at the EEEE locus but is not conducted into the cell, can support glucose-induced insulin release in rat pancreatic islets. By analogy, Ca^{2+} bound at the EEEE locus of the channel, which is tightly restricted by the ionic radius, would be sufficient to support insulin secretion prior to entry into the cells. Co-localization of the VGCC next to vesicles at release sites and the ability of the channel to bind and respond to Ca^{2+} make this proposition attractive.

A conformational coupling between the L-type channel and the exocytotic proteins appears to be the mechanistic

basis for excitation−secretion coupling in pancreatic cells. It allows for conformational changes at a channel associated with synaptotagmin, SNAP-25, and syntaxin 1A (the excitosome complex) to be rapidly transmitted to docked vesicles to elicit exocytosis. Such an arrangement enables the tight coupling between the triggering event and fusion of docked vesicles. These results imply that synaptotagmin(s) and other intracellular proteins that interact with Ca^{2+} act further downstream in the secretion pathway.

The observation that La^{3+} supports exocytosis in two different cell types, one involving a hormone release and the other involving a neurotransmitter release, implies that our model of the VGCC as a primary Ca^{2+}-sensor protein might be a general phenomena of many cell types where stimulus−secretion coupling is mediated by the calcium channel.

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