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The Inhibitors of Protein Acylation, Cerulenin and Tunicamycin, Increase Voltage-Dependent Ca^{2+} Currents in the Insulin-secreting INS 832/13 Cell

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Abstract

As it has been suggested that protein acylation plays a role in nutrient stimulus-secretion coupling in the pancreatic β -cell, we examined the insulin secreting INS 832/13 β -cell line for evidence that protein acylation was involved. The perforated whole-cell configuration was employed to voltage-clamp INS 832/13 cells. Voltage pulses were applied and Ca^{2+} -currents measured in the presence and absence of the protein acylation inhibitors cerulenin and tunicamycin. Both inhibitors enhanced the peak amplitude of $I_{\text{Ca,L}}$. Both increased the peak inward current in the range between -40 and $+30$ mV and shifted the apparent maximum current by 10 mV in the hyperpolarizing direction without affecting the activation threshold of -40 mV. The two drugs had qualitatively and quantitatively similar effects. Steady state activation curves revealed that cerulenin and tunicamycin shifted the activation curves in the hyperpolarization direction. Activation time constants were significantly reduced in the presence of both drugs. The Ca^{2+} charge influx was increased by the drugs at all potentials tested. In contrast to these effects on the L-type Ca^{2+} channel, the two inhibitors of protein acylation had no effect on the ATP-sensitive K^{+} channel. The results suggest that protein acylation exerts a tonic inhibitory effect on L-type Ca^{2+} channel function in the insulin secreting β -cell.

1. Introduction

Acylation is important for the integrity and action of many proteins and their cellular functions [1–6]. Several proteins involved in the control of insulin secretion are known to be acylated. These include heterotrimeric [7] and low molecular weight [8] G proteins, Ca^{2+} channels [9], and SNAP-25 and synaptotagmin that are involved in exocytosis *per se* [10,11]. Protein S-acyltransferase (PATs) activity is likely the dominant mechanism for cellular thioacylation, linking fatty acyl CoAs (commonly palmitoyl CoA) to cysteines with a thioester linkage [1, 12]. However, slow non-enzymatic protein acylation also occurs. In recent years some PAT activities have been identified [13]. As protein acylation is a reversible process with dynamic cycles of acylation and deacylation [8,14] it is capable of playing a major role in signal transduction. It has been suggested that protein acylation plays a signaling role in the control of glucose-stimulated insulin secretion [15–19] and in the action of inhibitors of secretion such as norepinephrine [20]. In view of the importance of Ca^{2+} -channels in the stimulation of insulin secretion [21–26] and the observation that Ca^{2+} channels in bovine chromaffin cells are

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acylated [9], we studied the effects of two inhibitors of protein acylation, cerulenin and tunicamycin, on the activity of L-type Ca^{2+} -channels in the β -cell line INS 832/13. They had similar effects to increase Ca^{2+} -currents over the entire voltage range tested. This suggests that protein acylation of the channels exerts a tonic inhibitory effect and may have a dynamic physiological role.

2. Materials and Methods

2.1 Cell preparation

INS 832/13 cells, a kind gift from Dr. C.B. Newgard, were cultured in RPMI 1640 medium supplemented with 11.1 mM glucose, 10% (vol/vol) heat inactivated fetal calf serum, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were plated on 35mm dishes and used one day after plating. Before electrophysiological measurements, the cells were pre-incubated in KRB buffer at 37°C for 25 minutes, in the presence or absence of 100 μM Cerulenin or 12 μM tunicamycin. These concentrations were selected because of their known effectiveness in blocking protein acylation.

2.2 Electrophysiological measurements of Ca^{2+} and KATP channel currents

The perforated whole-cell configuration was employed to voltage-clamp INS 832/13 cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Sarasota, USA), and “fire-polished” to obtain a tip resistance of 2 to 5 megohm when filled with intracellular solution. The tip of the patch pipette was first filled with Amphotericin B-free pipette solution by capillary action and then backfilled with Amphotericin B-containing internal solution. The final Amphotericin B concentration was 200 $\mu\text{g/ml}$. The reference potential for all experiments was the zero current compensated before the establishment of the seal. The electrode resistance was monitored by applying a 1 mV square pulse of 5 ms duration at 10 KHz. Once a high resistance seal ($> \text{G } \Omega$) was formed between the recording pipette and the cell membrane, the access resistance was continuously monitored. The recording was performed only when the access resistance reached a value of $< 25 \text{ M } \Omega$ and remained constant throughout the experiment. An EPC-7 amplifier (List Electronics) driven by Axograph 4.6 software in conjunction with an A/D, D/A board (DigiData 1322A interface, Axon Instruments Inc.), was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. Current signals, after compensation for whole-cell capacitance, series resistance and liquid junction potential were low-pass filtered at 2.3 KHz and digitized at 20 KHz prior to being stored on the computer hard disk. $\text{I}_{\text{Ca,L}}$ was always recorded in 2.6 mM Ca^{2+} -containing extracellular solution and I_{KATP} was elicited by low glucose extracellular solution (Glucose 0.2 mM). Both $\text{I}_{\text{Ca,L}}$ and I_{KATP} were measured once the current amplitude had stabilized. In order to avoid possible cell-cell coupling artifacts, cells that were not in contact with others were selected for the experiments. Under the perforated whole-cell configuration, $\text{I}_{\text{Ca,L}}$ and I_{KATP} did not show significant run down throughout the recordings. Both cerulenin and tunicamycin were dissolved in DMSO prior to dilution in KRB buffer or extracellular solution. INS 832/13 cells were preincubated in cerulenin or tunicamycin containing buffer at 37°C for 25–30 minutes before recordings were made. Cells were superfused with extracellular solutions containing or free of the two inhibitors during the recording periods by using a gravity flow system with a rate of 1.5–2 ml/min. All recordings were made at room temperature. Both cerulenin and tunicamycin experiments were compared with DMSO control experiments.

2.3 Solutions

For recordings of $\text{I}_{\text{Ca,L}}$, the solutions were (in mM) as follows, external NaCl 118, TEA-Cl 20, TTX 0.1, KCl 5.6, MgCl_2 1.2, CaCl_2 2.6, HEPES 10, Glucose 2.8, pH 7.4 with NaOH; internal CsCl 50, Cs_2SO_4 40, NaCl 10, KCl 10, MgCl_2 1.2, HEPES 10, pH 7.3 with CsOH. For

recordings of I_{KATP} , the solutions were as follows (in mM), external NaCl 138, TTX 0.1, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 10, Glucose 0.2 mM, pH 7.4 with NaOH; internal KCl 60, K_2SO_4 40, NaCl 10, $MgCl_2$ 1.2, HEPES 10, pH 7.3 with KOH. The composition of the KRB buffer (in mM) was NaCl 128.8, KCl 4.8, KH_2PO_4 -1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 5.0, HEPES 10, Glucose 2.8, pH 7.4 with NaOH.

2.4 Data analysis

Igor 5.0 (Wavemetrics) software was used for data analysis. Steady-state inactivation curves of $I_{Ca,L}$ were obtained using a double-pulse protocol. Pre-conditioning pulses of various amplitudes were applied (up to 30 mV, 2 s duration) before application of the test pulses (to 0 mV, 200 ms). The peak amplitude of the Ca^{2+} current evoked by each test pulse was measured in the presence or absence of the two drugs. Currents measured at each pre-conditioning potential were normalized to the peak current at the most hyperpolarized pre-conditioning potential and displayed as a function of pre-conditioning potentials. The resulting steady-state inactivation data were fitted with the Boltzmann equation:

$I_{peak}/I_{peak,max} = 1/1 + \exp[(V - V_{1/2})/k]$ where V is the preconditioning potential, $V_{1/2}$ represents the potential corresponding to the half-inactivation point, and k is the slope of the inactivation curve. Activation curves were derived from the current-voltage relationships and fitted with the following Boltzmann equation:

$I_{peak}/I_{peak,max} = 1/1 + \exp[-(V - V_{1/2})/k]$ where V represents the test potential, $V_{1/2}$ is the half-activation potential and k is the slope factor for the activation curve.

2.5 Statistical analysis

Data were averaged across cells and presented as means $\pm$ SEM. Statistical analysis was performed using Student's t test. The difference between treatment groups was considered to be significant when $p < 0.05$.

3. Results

3.1 Effects of the protein acylation inhibitors, cerulenin and tunicamycin, on $I_{Ca,L}$

Fig. 1 illustrates the effects of 100 μ M cerulenin and 12 μ M tunicamycin on $I_{Ca,L}$ in INS 832/13 cells. In these experiments, the Ca^{2+} currents were isolated by blocking K^+ currents with internal Cs^+ and external TEA, and by blocking possible Na^+ currents with TTX. Fig. 1A and Fig. 1B present examples of the Ca^{2+} current traces obtained under control conditions and in the presence of cerulenin or tunicamycin. The $I_{Ca,L}$ was elicited by 100 ms depolarizing pulses between -60 and 50 mV from a holding potential (V_h) of -70 mV in steps of 10 mV. Fig. 1A and Fig. 1B were from two data sets corresponding to the cerulenin and tunicamycin experiments. Both cerulenin and tunicamycin enhanced the peak amplitudes of $I_{Ca,L}$ and these effects were sensitive to the L-type Ca^{2+} channel blocker nifedipine. In the presence of 10 μ M nifedipine, at a voltage of 10 mV, the control $I_{Ca,L}$ was suppressed by 76% in the cerulenin experiments, and by 82%, in the tunicamycin experiments. In the presence of nifedipine, neither drug had any significant effect on the Ca^{2+} currents. With nifedipine the currents were 2.6 ± 0.2 pA/pF (control, $n = 4$) and 2.4 ± 0.2 with 100 μ M cerulenin ($n = 5$); and 2.9 ± 0.2 pA/pF (control, $n = 4$) and 3.1 ± 0.3 pA/pF with 12 μ M tunicamycin ($n = 6$).

The current-voltage relationships (I - V curves) were constructed under control and test conditions and are shown in Fig. 1C and Fig. 1D. In control cells, the threshold for the activation of $I_{Ca,L}$ was -40 mV, and the maximum peak current appeared at 10 mV. Both cerulenin and tunicamycin significantly increased the peak inward current in the range between -30 and 30 mV, and shifted the apparent maximum by 10 mV in the hyperpolarizing direction without,

however, varying the threshold of -40 mV. Both cerulenin and tunicamycin amplified the $I_{Ca,L}$ to the same extent (approximately doubling the peak current), indicating that cerulenin and tunicamycin had the same ability to enhance $I_{Ca,L}$.

3.2 Effects of cerulenin and tunicamycin on the steady-state inactivation and activation curves of $I_{Ca,L}$

Steady-state inactivation provides information about the number of channels available for activation when the cell membrane has remained at a specific potential for an extended time period. Hence, it is particularly informative of the availability of active channels around the resting membrane potential where the cells spend most of their time.

Using a double-pulse protocol as described in *Materials and Methods*, the effects of cerulenin and tunicamycin on steady-state inactivation of Ca^{2+} channels were tested and the curves fitted by the Boltzmann equation. The data shown in Fig. 2A reveal a leftward shift of the voltage dependence of steady-state inactivation in the presence of $100\ \mu\text{M}$ cerulenin, i.e. the voltage-dependent inactivation of $I_{Ca,L}$ was augmented by treatment with cerulenin. In contrast, $12\ \mu\text{M}$ tunicamycin did not significantly affect the steady-state inactivation curve (Fig. 2B), although at the same concentration, it significantly enhanced the current density of $I_{Ca,L}$ by ~ 2 folds. The 50% inactivation potential evaluated by the Boltzmann equation was shifted, compared with the control condition, by 5.3 ± 0.6 mV ($n = 13$, $p < 0.01$), and by 0.8 ± 0.04 mV ($n = 9$, N.S.) by $100\ \mu\text{M}$ cerulenin and $12\ \mu\text{M}$ tunicamycin, respectively. Furthermore, the slope was significantly steeper in the presence of $100\ \mu\text{M}$ cerulenin (control, $k = -5.7 \pm 0.6$ mV, $n = 28$; cerulenin, $k = -7.4 \pm 0.6$ mV, $n = 13$, $p < 0.01$). The slope factor was not significantly changed by $12\ \mu\text{M}$ tunicamycin (control, $k = -7.1 \pm 0.5$ mV, $n = 21$; tunicamycin, $k = -6.3 \pm 0.4$ mV, $n = 9$, N.S.). As the lack of the effect of tunicamycin on the inactivation curve at the concentration of $12\ \mu\text{M}$ could be due to too low a concentration, the experiments were repeated using $36\ \mu\text{M}$ tunicamycin. At this higher concentration, tunicamycin induced a rightward shift of the inactivation curve, Fig. 2B. In the presence of $36\ \mu\text{M}$ tunicamycin, the 50% inactivation potential evaluated by the Boltzmann equation was shifted, compared with the control by 3.9 ± 0.2 mV ($n = 16$, $p < 0.01$), and the slope factor was not significantly changed (control, $k = -7.1 \pm 0.5$ mV, $n = 21$; tunicamycin, $k = -7.3 \pm 0.5$ mV, $n = 16$, N.S.). When applied at the lower concentration of $3\ \mu\text{M}$, tunicamycin had no effect (data not shown). Thus cerulenin and tunicamycin have opposite effects on the inactivation of Ca^{2+} currents leading to the conclusion that these effects on inactivation are not related to protein acylation but are more likely due to non-specific effects of the drugs.

The activation curves derived from the I–V curves in Fig. 1C and Fig. 1D were fitted with the Boltzmann equation, Fig. 2A and Fig. 2B. Both cerulenin and tunicamycin slightly but significantly shifted the activation curves in the hyperpolarization direction. The 50% activation potential was shifted, compared with the control condition, by 5.2 ± 0.4 mV ($n = 13$, $p < 0.01$), and by 4.3 ± 0.2 mV ($n = 9$, $p < 0.01$) by cerulenin and tunicamycin, respectively. The slope factors (k) were not significantly changed by cerulenin (control, $k = 6.0 \pm 0.2$ mV, $n = 28$; cerulenin, $k = 5.4 \pm 0.4$ mV, $n = 13$, N.S.) nor by tunicamycin (control, $k = 5.6 \pm 0.2$ mV, $n = 17$; tunicamycin, $k = 5.1 \pm 0.3$ mV, $n = 9$, N.S.).

3.3 Effects of cerulenin and tunicamycin on $I_{Ca,L}$ kinetics

The Ca^{2+} current traces evoked by depolarization pulses exhibited two phases with different kinetics, the initial fast activation phase and then the relatively slow decaying phase. Activation time constants (τ_1) determined by mono-exponential fitting for $I_{Ca,L}$ traces evoked at 0 mV were significantly smaller in the presence of $100\ \mu\text{M}$ cerulenin and $12\ \mu\text{M}$ tunicamycin ($\tau_1 = 0.50 \pm 0.04$ ms, $n = 13$, $p < 0.05$, and $\tau_1 = 0.71 \pm 0.06$ ms, $n = 9$, $p < 0.05$), respectively, compared with the control values ($\tau_1 = 0.71 \pm 0.05$ ms, $n = 28$ and $\tau_1 = 0.85 \pm 0.15$ ms, $n =$

17). This effect was consistently present at all voltages tested, Fig. 3A and Fig. 3B. Thus, the time needed for $I_{Ca,L}$ to reach its peak value after a depolarizing stimulus was shorter in the presence of cerulenin or tunicamycin, reflecting a faster entry of $I_{Ca,L}$ channels to their open state. This may explain the increased peak current amplitude observed in cerulenin- and tunicamycin-treated conditions. The rate of current decay was described by a time constant (τ_2) obtained by exponentially fitting the decaying phase. Cerulenin significantly affected the velocity of current decline over all potentials tested, Fig. 3C, i.e., at the depolarizing potential of 0 mV, τ_2 was 37.4 ± 2.9 ms (control, $n = 28$) and 29.6 ± 1.9 ms (cerulenin, $n = 13$, $p < 0.05$). However, tunicamycin did not significantly change τ_2 at any potential tested, Fig. 3D.

3.4 Effects of cerulenin and tunicamycin on Ca^{2+} charge influx

The free Ca^{2+} that transfers into the cell during channel opening has biological significance because $[Ca^{2+}]_i$ is so tightly related to exocytosis in insulin-secreting cells. It was therefore important to estimate the total Ca^{2+} influx through the channels that were altered by cerulenin and tunicamycin. The charge quantity was calculated by integrating the inward current traces elicited by each of the depolarizing pulses. At 0 mV, both 100 μ M cerulenin and 12 μ M tunicamycin significantly increased Ca^{2+} influx (control versus cerulenin and tunicamycin, respectively, $n = 20$ and 12, 54.1 ± 1.3 fC/pF and 59.5 ± 1.8 fC/pF; cerulenin, $n = 9$, 69.4 ± 4.5 fC/pF, $p < 0.05$; tunicamycin, $n = 7$, 87.2 ± 5.8 fC/pF, $p < 0.01$). Increased Ca^{2+} influx was seen at all voltages tested, Fig. 4.

3.5 Effects of cerulenin and tunicamycin on the ATP-sensitive K^+ channel

It is well known that the mechanism of exocytosis of insulin granules involves the regulation of $I_{Ca,L}$ by the ATP-sensitive K^+ channel (KATP). Closure of the KATP channels and inhibition of I_{KATP} depolarizes the membrane and activates $I_{Ca,L}$. The rapid increase of $[Ca^{2+}]_i$ due to the channel opening triggers the release of insulin. The results presented so far demonstrate that acylation inhibitors modulated $I_{Ca,L}$ and changed the amount of Ca^{2+} charge influx in line with the suggestion that protein acylation could be involved in the control of glucose-stimulated insulin secretion. As a control study, the influence of cerulenin on I_{KATP} was investigated. Cells were held at -70 mV and stepped for 200ms to potentials between -110 mV and 10 mV at 10 mV increments. The peak I_{KATP} currents measured when cells were stepped from -70 to -90 mV were averaged and compared between control and cerulenin-treatment groups. The analysis showed that 100 μ M cerulenin did not induce any modification of I_{KATP} (control, -2.5 ± 0.3 pA/pF, $n = 18$; cerulenin, -2.6 ± 0.3 pA/pF, $n = 17$, $p > 0.05$).

4. Discussion

The idea that protein acylation could be involved in stimulus-secretion coupling for glucose-stimulated insulin secretion was first suggested in 1989 [15]. Subsequent studies proposed that protein acylation might be involved in the KATP channel-independent amplification pathway responsible for time-dependent potentiation (TDP) and the second phase of glucose-stimulated insulin secretion [17,18]. Evidence in support of the idea was based originally on the malonyl CoA/long chain acyl CoA hypothesis that postulates a glucose-induced increase in the concentration of long chain acyl CoA in the cytosol [15], and subsequently on the use of cerulenin, a fungal antibiotic and well-known inhibitor of protein acylation [17,18]. The essential findings with cerulenin were that it had no effect on basal insulin secretion but had a powerful inhibitory effect on both phases of glucose-stimulated insulin release. The effect on the first phase suggested that the operation of the KATP channels, voltage-dependent Ca^{2+} channels, or exocytosis *per se*, has been compromised. However, cerulenin had no effect on the response to a depolarizing concentration of KCl, so that the Ca^{2+} channels were operating normally in response to depolarization and the subsequent exocytotic response to elevated $[Ca^{2+}]_i$ was also unaffected. It is now known that cerulenin inhibits glucose oxidation, glucose

utilization, and oxygen consumption and reduces ATP levels [27]. Thus the effects of cerulenin to inhibit glucose-stimulated insulin secretion could be due to inhibition of protein acylation critical to stimulus-secretion coupling, to inhibition of protein acylation if the latter is essential at some point in the metabolism of glucose, to the impaired glucose metabolism whatever the cause, or simply to what would be described as a “non-specific” effect. However, while inhibition of glucose-stimulated insulin secretion by cerulenin can no longer be considered evidence in favor of a role for protein acylation in stimulus-secretion coupling, the original hypothesis [15] is still valid. Protein acylation is important for the integrity and action of many proteins including several that have roles in the control of insulin secretion, e.g. heterotrimeric G proteins [1], Ca^{2+} channels [9], and the SNARE proteins SNAP-25 [10] and synaptotagmin [11]. In view of this, it is more than likely that there are roles for protein acylation in the control of insulin release. Their importance remains to be determined. The first attempt to seek direct evidence for a role of protein acylation in insulin secretion coupling was by Yamada et al [19] who identified ^3H -palmitoylated proteins by SDS-PAGE and two-dimensional gel electrophoresis after labeling rat pancreatic islets and INS-1 cells with ^3H -palmitate. Four major palmitoylated bands were detected. The palmitoylation in all four was reduced by high glucose. The reduction was ascribed to the effect of glucose to increase long chain acyl-CoA levels and thereby decrease the specific activity of the ^3H -palmitic acid in the cell. Further analysis of their data suggested a functional role for a palmitoylated 24-kDa doublet in glucose-stimulated insulin secretion. It should also be noted that the direct application of long chain acyl CoA amplified the secretory response to Ca^{2+} when capacitance was used as a measure of exocytosis and that the amplification was blocked by cerulenin [16]. Interestingly, there is evidence that protein acylation may be involved in the inhibition of insulin secretion by the physiological inhibitors norepinephrine, somatostatin, galanin and PGE_2 [20]. This suggests that in the same way, for example, that signaling through protein phosphorylation can have stimulatory or inhibitory effects within the cell depending upon their targets, so protein acylation may do likewise.

Cerulenin, a fungal antibiotic that inhibits protein acylation [28,29], has been used to indicate that protein acylation is involved in activities as diverse as Ca^{2+} channel modulation [9], internalization [29,30], the stimulation of insulin release [16–19] and more recently, the inhibition of insulin secretion [20]. In the present study, we show that cerulenin enhanced the peak amplitude of $I_{\text{Ca-L}}$ in the range of -40 to $+30\text{mV}$ and shifted the maximum of the current-voltage relationship towards more negative potentials without changing the activation threshold for $I_{\text{Ca-L}}$. As the possibility exists that the effect of cerulenin was not due to inhibition of protein acylation and might have been due to non-specific effects, we also tested tunicamycin, another inhibitor of protein acylation that is structurally different from cerulenin [9,31,32]. Tunicamycin, like cerulenin, increased the peak amplitude of $I_{\text{Ca-L}}$ at all test potentials and shifted the maximum of the I-V curves in the same direction as cerulenin. It might be argued that the effects of cerulenin and tunicamycin observed here are not only due to their action on $I_{\text{Ca-L}}$, but rather the consequence of the recruitment of other types of Ca^{2+} channels, e.g. N-type Ca^{2+} channels. However, in the presence of the L-type Ca^{2+} channel blocker nifedipine neither tunicamycin nor cerulenin had any effect on the Ca current. This excludes the possibility that the increased $I_{\text{Ca-L}}$ recorded in our study was due to the activation of Ca^{2+} channels other than the L-type.

Based on the I–V curves shown in Fig. 1 and on the dual-pulse protocol, the effects of cerulenin and tunicamycin on the curves of steady-state activation and inactivation were compared. Both cerulenin and tunicamycin shifted the activation curves to more negative potentials. Cerulenin, but not tunicamycin, slightly but significantly shifted the inactivation curve to more negative potentials. Because tunicamycin (at $12\text{ }\mu\text{M}$) was as effective or more effective than cerulenin (at $100\text{ }\mu\text{M}$) in every respect except that it did not change the inactivation curve, it is likely that this effect of cerulenin is a non-specific effect and not due to inhibition of protein acylation.

In order to be sure that we had not missed the effect of tunicamycin by using too low a concentration, the experiments were repeated with 36 μM tunicamycin. At this higher concentration there was an effect of tunicamycin. However, it was in the opposite direction to that induced by cerulenin, i.e. to more positive potentials. Finally, to check whether this “opposite” effect of tunicamycin was obscuring a shift to more negative potentials we used a lower concentration of tunicamycin (3 μM). At this concentration, as at 12 μM , tunicamycin had no effect. As the two inhibitors had different effects on the inactivation curves we presume that the effects are not due to inhibition of protein acylation but due to non-specific effects of the drugs. We next studied the effects of cerulenin and tunicamycin on activation and deactivation kinetics of $I_{\text{Ca-L}}$. For the control experiment, the activation and deactivation kinetics assessed from whole-cell current traces were in the order of microseconds, which were exceedingly fast. This is in sharp contrast to the relatively slow kinetics of the L-type Ca^{2+} channel in cardiac myocytes and sensory neurons [33,34]. However, the activation time constants were similar to those obtained from $I_{\text{Ca-L}}$ in mouse pancreatic β – cells [35]. The kinetic analysis reveals that both cerulenin and tunicamycin accelerate the Ca^{2+} current activation by significantly shortening the time constant of the initial activating phase. Cerulenin was also found to speed up the current deactivation while tunicamycin (12 μM), as noted earlier, lacked any effect on the inactivation curves. The functional consequence of $I_{\text{Ca-L}}$ modulation by protein acylation would be expected to change the Ca^{2+} influx that is tightly related to insulin secretion in the β -cell. When the charge influx was estimated by integrating the current traces over the entire period of the depolarization pulse, cerulenin and tunicamycin increased the amount of Ca^{2+} flowing through the L-type Ca^{2+} channels by ~ 1.3 and ~ 1.5 fold, respectively.

When the effects of cerulenin and tunicamycin on K_{ATP} channels were investigated, no changes were detected. This is further support for the idea that most of the changes by cerulenin and tunicamycin on $I_{\text{Ca-L}}$ are specific and related to inhibition of protein acylation. While this work does not demonstrate directly that the Ca^{2+} channel is acylated there are data in the literature that Ca^{2+} channels containing the β 2a subunits are palmitoylated (9).

The fact that both cerulenin and tunicamycin enhanced Ca^{2+} currents through L-type voltage-dependent Ca^{2+} channels, suggests that protein acylation of the Ca^{2+} channels or some other protein that is interacting with the channel is responsible for restraining the amount of Ca^{2+} that passes into the β -cell on depolarization and that it does this at all levels of depolarization. It appears that a dynamic cycle of protein acylation and deacylation must be occurring continually. If this were not the case, e.g. if the channel or interacting protein were permanently acylated during synthesis or trafficking, then cerulenin and tunicamycin would have no effect in the mature cell. In view of these findings, it is important to determine directly whether the β -cell Ca^{2+} channels are acylated, what controls the acylation, and its physiological role.

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Fig. 1.

Cerulenin and tunicamycin potentiated the current density of $I_{Ca,L}$ in INS 832/13 cells. Fig. 1A and Fig. 1B show original recordings of perforated whole-cell $I_{Ca,L}$ elicited with 200 ms rectangular pulses from V_h of -70 mV to test potentials in the range of -60 to +50 mV (see schematic diagram between figures 1A and 1B), measured under control conditions and in the presence of 100 μ M cerulenin (control cells $n=13$, test cells $n=13$), and 12 μ M tunicamycin (control cells $n=17$, test cells $n=13$). The current-voltage relationships in Fig. 1C and Fig. 1D were constructed according to the datasets for the cerulenin and tunicamycin experiments, respectively. Data points are means $\pm$ SEM.

Fig. 2.

Cerulenin and tunicamycin differentially affect steady-state activation and inactivation of $I_{Ca,L}$. Data presented in Fig. 2A and Fig. 2B were obtained from data sets for cerulenin and tunicamycin experiments, respectively. Data were fitted by the Boltzmann equation. Cerulenin shifted both steady-state activation and inactivation curves to the more negative potential, Fig. 2A. 12 μ M Tunicamycin shifted the steady-state activation curve in the same direction as did cerulenin, but had no effect on the steady-state inactivation curve. When the concentration of tunicamycin was increased to 36 μ M, the steady-state inactivation curve was significantly shifted to more positive potentials Fig. 2B. All data points are presented as means $\pm$ SEM.

Fig. 3.

The effects of cerulenin and tunicamycin on the kinetic of $I_{Ca,L}$. The upper figures describe the effects of cerulenin (Fig. 3A) and tunicamycin (Fig. 3B) on the time constants (τ_1) of $I_{Ca,L}$ activation. The lower figures summarize the effects of cerulenin (Fig. 3C) and tunicamycin (Fig. 3D) on the time courses (τ_2) of $I_{Ca,L}$ de-activation. τ_1 and τ_2 were determined by fitting current traces with single exponential functions and measured over the voltage range of $-20 \sim +20$ mV. Data bars are mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4. Cerulenin and tunicamycin enhanced the total Ca^{2+} influx at all voltages tested. Ca^{2+} influx was calculated by integrating the current traces over time and normalized to cell size. Data bars are mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$.