Differential modulation of L-type calcium channel subunits by oleate

Yingrao Tian,1,2 Richard F. Corkey,2 Gordon C. Yaney,2 Paula B. Goforth,1 Leslie S. Satin,1 and Lina Moitoso de Vargas2

1Department of Pharmacology and Toxicology, Virginia Commonwealth University Medical Center, Richmond, Virginia; and 2Division of Molecular Medicine and Obesity Research Center, Boston Medical Center, Boston, Massachusetts

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Tian Y, Corkey RF, Yaney GC, Goforth PB, Satin LS, Moitoso de Vargas L. Differential modulation of L-type calcium channel subunits by oleate. Am J Physiol Endocrinol Metab 294: E1178–E1186, 2008. First published April 22, 2008; doi:10.1152/ajpendo.90237.2008.—Nonesterified fatty acids such as oleate and palmitate acutely potentiate insulin secretion from pancreatic islets in a glucose-dependent manner. In addition, recent studies show that fatty acids elevate intracellular free Ca2+ and increase voltage-gated Ca2+ current in mouse β-cells, although the mechanisms involved are poorly understood. Here we utilized a heterologous system to express subunit-defined voltage-dependent L-type Ca2+ channels (LTCC) and demonstrate that β-cell calcium may increase in part from an interaction between fatty acid and specific calcium channel subunits. Distinct functional LTCC were assembled in both COS-7 and HEK-293 cells by expressing either one of the EYFP-tagged L-type α-subunits (β-cell Cav1.3 or lung Cav1.2) and ERFP-tagged islet β-subunits (β2a or β3). In COS-7 cells, elevations in intracellular Ca2+ mediated by LTCC were enhanced by an oleate-BSA complex. To extend these findings, Cav2+ was measured in LTCC-expressing HEK-293 cells that revealed an increase in peak Ca2+ current within 2 min after addition of the oleate complex, with maximal potentiation occurring at voltages 0 mV. Both Cav1.3 and Cav1.2 were modulated by oleate, and the presence of different auxiliary β-subunits resulted in differential augmentation. The potentiating effect of oleate on Cav1.2 was abolished by the pretreatment of cells with triacsin C, suggesting that long-chain CoA synthesis is necessary for Ca2+ channel modulation. These results show for the first time that two L-type Ca2+ channels expressed in β-cells (Cav1.3 and Cav1.2) appear to be targeted by oleate (1, 15) might fulfill this requirement. The most direct evidence comes from studies showing that short-term exposure of pancreatic islets (11, 48, 64) or insulin-secreting cells (18) to FFA potentiates glucose-stimulated insulin secretion (GSIS). Elevation of intracellular Ca2+ through voltage-dependent calcium channels (VDCC) has long been established as an important and necessary signal for GSIS from pancreatic β-cells (2, 8, 33, 45, 55, 56, 66). Although many types of VDCC are expressed in pancreatic β-cells, L-type VDCC (LTCC) have emerged as major participants in insulin secretion (16, 32, 40, 58). Several studies indicate that FFA modulate LTCC in different cell types (38, 65), including pancreatic mouse β-cells (64) and enteroendocrine cells (38, 60). FFA induces an increase in intracellular free Ca2+ concentration ([Ca2+]i), possibly as a result of increased calcium influx through LTCC, and it has been shown that palmitate increases whole cell Ca2+ currents solely via LTCC activation in both β- and α-cells of pancreatic mouse islets (3, 42). In addition, our own work in INS-1 cells shows that exogenous oleate increases intracellular Ca2+ following cell depolarization and potentiates whole cell Ca2+ currents (Yaney GC, Moitoso de Vargas L, and Satin LS, unpublished observations). The FFA-mediated effect in [Ca2+]i parallels the acute FFA-induced potentiation of GSIS, reflecting a similar glucose-dependent membrane depolarization.

Here we transiently transfected COS-7 and HEK-293 cells with different combinations of Ca2+ channel subunits to investigate the role of individual LTCC subunits as potential oleate targets. Our data show that in COS-7 cells oleate enhanced the amplitude of LTCC-dependent elevations in [Ca2+]i. In addition, in LTCC-expressing HEK-293 cells, the whole cell patch clamp technique revealed that oleate acutely increased peak Ca2+ current. The potentiation was voltage-dependent and was blocked by the inhibition of long-chain acyl-CoA formation. Moreover, although both LTCC α-subunits, Cav1.3 and Cav1.2, were potentiated by oleate, β-subunits imparted an additional level of regulation on this effect in Cav1.2-containing channel multimers. The results are discussed with regard to the potentiating actions of fatty acids on glucose-dependent insulin secretion in pancreatic islets of Langerhans.
MATERIALS AND METHODS

Construction and preparation of plasmids and recombinant adenovirus. Plasmids pEYFP-α1C and pEYFP-β1D were constructed using standard molecular biology protocols by inserting the DNA coding for the rabbit lung α1C (Cav1.2) (5) or the HIT-T15 long COOH-terminal α1T (Cav1.3) isoform (Moitozo de Vargas L, unpublished observations) into pEYFP-C1 (Clontech) to generate in-frame fusions between the amino terminus of α1C and α1D and the carboxyl end of the yellow variant of the green fluorescent protein from Actinomycetae Victoria (EYFP; Clontech), EYFP-α1C, and EYFP-α1D, respectively. Similarly, we generated pβ2-ERFP, pmutβ2-ERFP, and pβ3-ERFP to produce functional fusion proteins at the COOH-terminal end of each islet β-subunit and the amino terminus of the enhanced JRed protein (ERFP, Evrogen). Constructs were confirmed by restriction enzyme and DNA-sequencing analyses of relevant regions.

Recombinant, replication-deficient type 2 adenoviruses (rAd) containing either β2-ERFP, mutβ2-ERFP, or β3-ERFP at the viral E1 region were produced using the two-cosmid system (63). Briefly, the genes coding for either β2-ERFP, mutβ3-ERFP, or β3-ERFP were subcloned into an adenoviral shuttle vector, pLPEM9, a pLPE (63) derivative containing the cytomegalovirus promoter and an SV40 poly(A) signal (Moitozo de Vargas L, unpublished observations) from which genomic adenoviral cosmids were subsequently obtained. rAd-expressing β2-ERFP, mutβ2-ERFP, or β3-ERFP (Ad β2-ERFP, Ad mutβ2-ERFP, Ad β3-ERFP, respectively) were transfected into HEK-293 cells with the isolated adenoviral cosmids. The correct DNA inserts were verified and confirmed in the rAd genome by polymerase chain reaction and restriction enzyme analyses, and protein expression was demonstrated by imaging of β2-ERFP, mutβ2-ERFP, or β3-ERFP-transduced COS-7 cells prior to viral amplification and CsCl purification as described (17).

Transduction/transfection of COS-7 cells. COS-7 and HEK-293 cells were acquired from American Type Culture Collection and grown using standard protocols. COS-7 cells cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin were plated into 35-mm poly-d-lysine-coated glass bottom microwell dishes (MatTek) containing 10% FBS and 1% penicillin-streptomycin were plated into 35-mm poly-d-lysine-coated glass bottom microwell dishes (MatTek) at ~0.5 × 10^6/ml. Following overnight culture, cells were treated with virus (Ad β2-ERFP, Ad mutβ2-ERFP, or Ad β3-ERFP) at a multiplicity of infection of 1–5 × 10^7–8 particles/cell for 1 h at 37°C, followed by transient transfection of 1 mg of plasmid DNA (pEYFP-α1C or pEYFP-β1D) using FuGENE 6 (Roche) according to the manufacturer’s instructions. After 3 h at 37°C, more DMEM medium was added and the cells were further incubated for 48 h. COS-7 passage number was not found to affect the expression of VDCC subunits, an observation that was also verified in HEK-293 cells.

Fura 2 loading and measurements of Ca^{2+}. Fura 2-AM (Molecular Probes) was used for ratiometric measurement of [Ca^{2+}], values using excitation at 340 and 380 nm and emission at 510 nm, as described previously (27). Briefly, Ca^{2+} measurements from single LTCC-expressing or control COS-7 cells were carried out after cells were loaded for 90 min at 37°C in the presence of 0.2% pluronic F-127 (Molecular Probes) with fura 2-AM (2 μM) in Krebs-Ringer-bicarbonate buffer containing 10 mM glucose, 2 mM CaCl2, and 0.05% BSA. After loading, the cells were washed, incubated in the same buffer without pluronic for 15 min, and imaged to select yellow fluorescent protein (YFP)- and red fluorescent protein (RFP)-expressing cells using a Zeiss IM 35 inverted microscope and a ×40 glycerin objective in a temperature-controlled cabinet heated to 37°C. A xenon lamp and a dual-excitation filter (51019 series) from Chroma were used to excite YFP and RFP differentially. A dual dichroic and emission filter pair from the same series (Chroma) allowed the selective imaging of YFP and RFP. Subsequently, intracellular fura 2 of selected cells was excited at 340 and 380 nm and emission signals (510 nm) recorded by a charge-intensified charge-coupled device camera. Images were collected at 8-s intervals. Data were acquired and analyzed using IonWizard software (IonOptix). The free Ca^{2+} concentration was calculated from the fluorescence ratio (25). A Kd of 224 nm/l for Ca^{2+} binding to free fura 2 was used in calculations.

Preparation of BSA-oleate complexes. An oleate-BSA complex was used, since most long-chain fatty acids in the circulation are bound to albumin with a free FFA concentration ranging from 0.01 to 10 μM (61). A complex of 20 mM oleate was made as follows. A 35% BSA stock was prepared in dH2O by very slowly adding fatty acid-free BSA (Sigma) in small aliquots with minimal stirring. The final concentration of BSA was determined by the solution’s absorbance at 280 nm minus that at 350 nm and assuming a molecular weight of 67,000. A 50-mM oleate stock was prepared in 4 mM NaOH using the sodium salt of oleate (Sigma). Combining 1 ml of 50 mM oleate stock and 1.5 ml of 4 mM BSA after heating both to 55°C results in 2.5 ml of 20 mM oleate at an oleate/BSA ratio of ~8:3:1. Because BSA has multiple binding sites for FFA, this FFA/oleate ratio provides an estimated concentration of 0.5% of free oleate in the oleate-BSA complex (52). The different oleate concentrations in the experiments reported here thus approximate the in vivo levels of unbound and FFA.

Culture and transfection of HEK-293 cells. HEK-293 cells were placed on glass coverslips in 35-mm petri dishes and cultured in MEM medium with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Cultures were kept at 37°C in an air-5% CO2 incubator. Cells were transiently transfected by the calcium phosphate precipitation method 30 h after plating using 0.5 μg of each construct. Following transfection, cells were washed with PBS for 10 min, which was then replaced with fresh medium supplemented with 10 mM MgCl2.

Electrophysiology. Twenty-four hours after transfection, HEK cells were visually selected for recording by yellow fluorescent protein fluorescence. Whole cell or perforated patch clamp recordings were performed with an Axopatch 1-D amplifier (Axon Instruments). Patch pipettes (4–6 Mohm) contained 114 mM Cs aspartate, 10 mM CsCl2, 4 mM Mg2ATP, 10 mM HEPES, and 1 mM EGTA with or without 0.15 mg/ml amphoterocin B (pH 7.2). The extracellular recording solution contained 115 mM NaCl, 3 mM CaCl2, 5 mM CsCl2, 1 mM Mg2Cl2, 10 mM HEPES, 1.1 mM glucose, and 0.05% BSA (pH 7.2). In a small subset of cells, CaCl2 was replaced by 3 mM BaCl2, which did not affect the degree of current potentiation by oleate. Recordings were performed at a holding potential of ~65 mV, and peak current was acquired at 0 μV unless otherwise indicated in the text.

Data analysis. Unless otherwise stated, data analysis and graphics were performed using IGOR Pro software, and statistics were performed using Prism3 software. Data are presented as means ± SE for the indicated number of experiments. Statistical significance between two means was evaluated using Student’s t-test.

RESULTS

Effect of oleate in [Ca^{2+}], of Cav1.3 or Cav1.2 plus β2-ERFP-expressing COS-7 cells. We established a heterologous system (COS-7 cells) to express differentially fluorescent-labeled α1- and β-subunit isoforms of L-type VDCC and performed intracellular [Ca^{2+}] measurements of fura 2-loaded single cells according to the procedures described in MATERIALS AND METHODS. A similar protocol has been described previously and validated (37). Parallel control experiments were performed with COS-7 cells lacking transgenes or expressing only one of the VDCC subunits (either EYFP-α1 or β2a-ERFP alone). The resting membrane potential of COS-7 cells, −31 mV, is comparable with that evoked by 7 mM glucose in native β-cells and which supports tonic activation of the Cav1.3-containing channel (67).

Under these conditions, an increase in extracellular [Ca^{2+}] would be expected to cause a concomitant rise in intracellular [Ca^{2+}] in COS-7 cells expressing functional Cav1.3-β2a Ca^{2+}-
channels. Intracellular [Ca$^{2+}$] was measured in single fura-2 loaded COS-7 cells under conditions of 2 mM (basal) or 5 mM extracellular Ca$^{2+}$ and in the presence or absence of the oleate-BSA complex. The dependence of the Ca$^{2+}$ changes observed in VDCC was confirmed using the L-type Ca$^{2+}$ channel agonist Bay K 8644, and the viability and integrity of control COS-7 cells were confirmed with 10 μM ATP, which releases intracellular Ca$^{2+}$ from internal stores (4).

Compared with control COS-7 cells, on average, [Ca$^{2+}$]$_{i}$ in Cav1.3-i$\beta_2$a-expressing COS-7 cells was elevated by 40% when bathed in 2 mM (basal) extracellular Ca$^{2+}$. Also, as shown in Fig. 1A, increasing extracellular Ca$^{2+}$ to 5 mM (CaCl$_2$) resulted in a rise in intracellular [Ca$^{2+}$] in Cav1.3-i$\beta_2$a-expressing COS-7 cells, but not in COS-7 lacking heterologously expressed LTCC subunits (Fig. 1B) or in those with i$\beta_2$a only (data not shown). Moreover, subsequent addition of the oleate complex further increased intracellular Ca$^{2+}$ in Cav1.3-i$\beta_2$a-expressing COS-7 cells, but not in control cells lacking channel expression. Oleate increased [Ca$^{2+}$]$_{i}$ by a mean of 41%, which took place within 2–8 min after its addition.

Effect of oleate on [Ca$^{2+}$]$_{i}$ in Cav1.3 or Cav1.2 plus mut i$\beta_2$a-expressing COS-7 cells. Previous work has shown that the NH$_2$-terminal cysteines (C3, C4) of rat brain i$\beta_2$a undergo palmitoylation, which confers distinct properties to the LTCC that result from its association with the \( \alpha_1 $-subunit. In particular, palmitoylation allows membrane localization and targeting of the \( \beta $-subunit independently of its association with \( \alpha $, shifts its voltage activation to more negative membrane potentials, affects prepulse facilitation and channel inactivation ki-

**Fig. 1.** Oleate increases intracellular Ca$^{2+}$ in single COS-7 cells expressing L-type voltage-dependent calcium channels (LTCC). Representative traces of changes in intracellular free calcium concentration ([Ca$^{2+}$]$_{i}$) in fura 2-loaded COS-7 cells as specified in MATERIALS AND METHODS in the presence of 10 mM glucose, 2 mM CaCl$_2$, and 0.05% BSA. Reagents were added sequentially at the times indicated as 500 μl bolus to the dish with a starting volume of 1 ml. A: COS-7 cells expressing Cav1.3-i$\beta_2$a for 48-h posttransfer of transgenes. B: COS-7 control cells in the absence of any heterologous expression of transgenes.

**Fig. 2.** Oleate increases intracellular Ca$^{2+}$ in single COS-7 cells expressing Cav1.3 plus mut i$\beta_2$a or i$\beta_3$. Representative traces, generated with 5-point moving average, of fura 2-loaded COS-7 cells expressing Cav1.3-mut i$\beta_2$a (A) and Cav1.3-i$\beta_3$ (B) imaged 48 h posttransgenes’ transfer in the presence of 10 mM glucose, 2 mM CaCl$_2$, and 0.05% BSA and monitored for [Ca$^{2+}$] changes after addition of 500 μl bolus of each reagent at the indicated times.

To determine whether the oleate-induced effect was restricted to Cav1.3-i$\beta_2$a, the studies were extended to the Cav1.2-i$\beta_2$a channel. We found that Cav1.2-i$\beta_2$a channels were functionally expressed in the COS-7 heterologous system and that the activity of these channels was also subject to enhancement by oleate (Fig. 3). Oleate in this case increased [Ca$^{2+}$]$_{i}$ by a mean of 107% 1.5 min after being added.
and C4 does not appear to be needed for oleate potentiation of LTCC channels, because the mutations did not interfere with the action of oleate (Figs. 2A and 3). Thus, oleate increased [Ca\(^{2+}\)]\(_i\) by a mean of 26% for Cav1.2-mut i\(\beta_2a\)-expressing cells and 45% for Cav1.3-mut i\(\beta_2a\)-expressing cells.

Effect of oleate on [Ca\(^{2+}\)]\(_i\) in Cav1.3 or Cav1.2 plus i\(\beta_2\) expressing COS-7 cells. To ascertain the independent role of i\(\beta_2a\) and i\(\beta_3\) (the predominant \(\beta\)-subunits expressed in \(\beta\)-cells) (31, 44, 68) in the fatty acid-mediated potentiation of intracellular Ca\(^{2+}\), we independently expressed either of \(\alpha_1\) subunit with rat i\(\beta_3\) (sequencing of its coding DNA revealed that it was identical to brain i\(\beta_3\): Moitoso de Vargas L, unpublished observations). The activation of Cav1.2 with i\(\beta_3\) in COS-7 cells, monitored as an increase in intracellular [Ca\(^{2+}\)], was not feasible from these experiments.

Effects of oleate on Ca\(^{2+}\) current carried by calcium channels Cav1.3 or Cav1.2 plus i\(\beta_2a\) and \(\alpha_2\delta\). To verify that the oleate-mediated rise in [Ca\(^{2+}\)]\(_i\) observed in LTCC-expressing COS-7 cells was the direct result of an increase in Ca\(^{2+}\) current due to FFA, and to obtain a more rigorous and quantitative analysis of the efficacy of different subunit contributions, we used a similar heterologous expression system (HEK-293 cells) to measure whole cell calcium currents in voltage-clamped HEK cells expressing Cav1.3 or Cav1.2 with i\(\beta_2a\) and \(\alpha_2\delta\). Cells were voltage-clamped to a holding potential of −65 mV, and peak inward calcium current was measured upon stepping to 0 mV. In HEK cells expressing Cav1.3, the application of 100 \(\mu\)M oleate-BSA increased peak Ca\(^{2+}\) current amplitude from −284 ± 54 to −318 ± 62 pA (\(n = 17\)), with a mean increase of 18.1 ± 8.3% (\(P < 0.05\); Fig. 4, A and B). Of 17 cells tested, oleate increased Ca\(^{2+}\) current amplitude in 12 cells, decreased it in three, and had no effect in the remaining two cells. The effect of oleate occurred within 2 min of its addition to the bath. Similarly, application of 100 \(\mu\)M oleate-BSA complex increased mean calcium current amplitude in cells expressing Cav1.2 from −282 ± 54 to −324 ± 59 pA.
Of 24 cells exposed to the FA, oleate increased current amplitude in 16 cells, decreased it in five, and had no effect in the remaining three cells. The maximum potentiation occurred within 1–4 min using this subunit combination. The mean oleate-elicited increase for Cav1.2 was $23.3 \pm 10.0\%$.

To test whether the action of oleate was voltage dependent, we constructed $\text{Ca}^{2+}$ current-voltage (I-V) curves for HEK cells expressing Cav1.2-i$\beta_{2a}$-$\alpha_2\delta$. Calcium currents were measured in the absence and presence of 20 or 100 $\mu$M oleate-BSA as membrane potential was changed from a standard holding potential of $-65$ mV to a series of potentials ranging from $-70$ to $+50$ mV. The application of 20 or 100 $\mu$M oleate increased $\text{Ca}^{2+}$ current in a voltage-dependent manner, with the largest enhancement occurring at more negative potentials. Thus, as shown in Fig. 5A, 20 $\mu$M oleate increased calcium current amplitude by $111\%$ at $-30$ mV, $74\%$ at $-20$ mV, $54\%$ at $-10$ mV, $41\%$ at 0 mV, and $39\%$ at $+10$ mV ($n = 10$). A similar result was obtained using 100 $\mu$M oleate, which significantly increased calcium current amplitude by $23\%$ at $-30$ mV, $37\%$ at $-20$ mV, $35\%$ at $-10$ mV, $12\%$ at 0 mV, and $4\%$ at $+10$ mV ($n = 14$; data not shown). Interestingly, 20 $\mu$M oleate increased current amplitude to a greater degree than 100 $\mu$M (compare Figs. 4B and 5B).

The effect of oleate on $\text{Ca}^{2+}$ channels possessing different $\beta$-subunits. Whole cell peak calcium currents were measured at 0 mV in HEK cells expressing either Cav1.2-i$\beta_{2a}$-$\alpha_2\delta$ or Cav1.2-i$\beta_3$-$\alpha_2\delta$ to test whether expression of $\beta_{2a}$ vs. $\beta_3$ differentially affected oleate action. In i$\beta_{2a}$-containing HEK cells, 20 $\mu$M oleate-BSA increased peak current amplitude from $-191 \pm 44$ to $-369 \pm 82$ pA ($n = 9$, $P < 0.01$; Fig. 5B), a $106 \pm 21\%$ increase. The expression of $\beta_3$ instead of $\beta_{2a}$ resulted in a more modest oleate-induced current enhancement (Fig. 5, C and D). In these cells, 20 $\mu$M oleate increased calcium current at 0 mV from $-241 \pm 48$ to $-277 \pm 54$ pA ($n = 8$, $P < 0.01$), an increase of only $16 \pm 2\%$ (note that change in scales shown in Fig. 5, B vs. D).
The effect of triacsin C on oleate-regulated calcium channel Cav1.2 with β_{2a} and α_{2δ}. To examine the possible role of long-chain CoA synthesis in oleate-induced enhancement of calcium channels, HEK-293 cells expressing Cav1.2-β_{2a}-α_{2δ} were preincubated in the presence of 96 μM triacsin C (TC) for 20 min. Although not effective for all isoforms, TC is a specific and potent competitive inhibitor of acyl-CoA synthetases, the enzymes that at the plasma membrane catalyze the initial step of activating FFA to acyl-CoAs, the substrates for both synthetic and oxidative pathways (14, 30). In β-cells, TC is reported to inhibit the production of long-chain CoAs (54). As shown in Fig. 6, TC treatment abolished oleate potentiation of L-type Ca^{2+} currents. After TC pretreatment, the application of 20 μM oleate had no potentiating effect on the calcium current I-V curves (Fig. 6A) and did not significantly increase peak calcium current measured at 0 mV, -196 ± 86 vs. -204 ± 93 pA (n = 7, not significant; Fig. 6B). These data suggest that long-chain CoA production is necessary for the oleate-induced increase of L-type calcium channel activity.

**DISCUSSION**

Previous reports indicate that short-term exposure to FFA potentiates GSIS, as well as [Ca^{2+}], in β-cells (15, 18, 48). Although the molecular and cellular mechanisms underlying these effects remain elusive, it appears that the FFA increase in [Ca^{2+}], likely involves both the influx of extracellular Ca^{2+} through LTCC as well as mobilization of Ca^{2+} from internal stores (23, 59). In this study, we employed heterologous expression systems to dissect potential FFA-LTCC interactions at the level of Ca^{2+} channel subunit. Both COS-7 and HEK-293 cells were well suited for our oleate studies because neither of these cell lines expresses receptors for either long-chain (Refs. 9 and 28, Fig. 1B, and Demerest K and Kuo G-H, personal communication) or short-chain fatty acids (10, 35). These receptors, whose tissue-specific expression was recently found, are not required for fatty acid uptake and transport because FFA readily and passively diffuse into and out of cells by the flip-flop mechanism (26). Because the two L-type
calcium channels that are predominantly expressed in pancreatic β-cells, Cav1.3 and Cav1.2, are pharmacologically indistinguishable, we expressed channels of predefined subunit composition to bypass any interference from the complex LTCC expression environment of native β-cells.

Our data indicate that in COS-7 cells acute application of the oleate-BSA complex results in a $[\text{Ca}^{2+}]_i$ rise that is dependent on the functional expression of LTCC and qualitatively independent of channel subunit composition (Figs. 1–3). Although this protocol provides a rapid and simple qualitative analysis of Ca$^{2+}$ channel functional expression and how it may be affected by external application of compounds (e.g., acute exposure to FFA), this approach does not allow a rigorous quantitative analysis of channel biophysical properties. Thus, to verify that FFA modulates LTCC’s characteristics, we expressed specific subunits of LTCC in HEK-293 cells and measured Ca$^{2+}$ currents. We show that short-term exposure to oleate increases peak current amplitude of Cav1.3 or Cav1.2 in conjunction with either β2a or β3. In our hands, the Cav1.2-β2a combination was most potentiated by oleate. The heterologous expression data taken together thus support the hypothesis that increased LTCC activity in turn mediates a rise in $[\text{Ca}^{2+}]_i$. In intact β-cells, this would in turn potentiate glucose-dependent insulin granule exocytosis.

Although Cav1.3 and Cav1.2 channels exhibit similar potentiation in response to a 100-μM complex of oleate-BSA, 18 and 23%, respectively, they differ significantly in their expression rates, 15 and 80%, respectively (data not shown). For this reason, a more rigorous analysis was conducted with the latter channel. The regulation of Cav1.2 channels by oleate appears to be dose dependent, as decreasing the concentration of the oleate-BSA complex to 20 μM results in a 106% increase in current amplitude, although the time courses of the initial (2 min) and peak (15 min) responses were delayed compared with those observed with the 100-μM oleate complex (30 s and 4 min, respectively). Analysis of the I-V curve of Cav1.2-β2a shows that oleate regulation of these channels is voltage dependent, with a greater enhancement occurring at more negative potentials. A more detailed study of the effects of oleate on Ca$^{2+}$ channel kinetics should further distinguish the mechanism involved.

Others have reported that palmitate exerts an effect on $[\text{Ca}^{2+}]_i$, and LTCCs in insulin-secreting cells (50, 64) and increases peak current amplitude by 23% (43). However, the present study is the first to report that oleate exerts a stimulatory effect on LTCC at the subunit level. Channels with Cav1.2, α5δ, and β2a or β3 all responded positively to oleate. Nevertheless, the effect of oleate on channels with β2a (106% ± 21% increase; Fig. 5B) was much greater than in channels with β3 (16% ± 2% increase; Fig. 5D), suggesting that the β-subunit plays a role in oleate regulation of channel activity. Unlike β3, which does not undergo lipid-mediated posttranslational modifications, β2a does, and an oleate-induced augmentation of the acylation status of this subunit may be the basis for the difference. Alternatively, the β3 subunit may interfere with the interaction between oleate and the α1-subunit. Thus, whereas both Cav1.3 and Cav1.2 can be modulated by oleate to a similar extent, the auxiliary β-subunit can exert further regulation of the effect. These findings may have implications for the oleate effect in β-cells, where both β2a and β3 are predominantly expressed, but their respective contributions for and associations with the Cav1.3 or Cav1.2 channels are not known. It has been reported recently that β3 may not participate or even be required for LTCC activity in β-cells, but rather, it may be important for Ca$^{2+}$ signaling involving internal Ca$^{2+}$ stores (4).

Recent findings indicate that two components comprise the FFA-mediated Ca$^{2+}$ increase in β-cells, mobilization from internal stores and influx through LTCC (23, 59). Some authors have concluded that release from intracellular stores through activation of GPR40, a G protein-coupled receptor for medium and long-chain FFA, precedes Ca$^{2+}$ influx through LTCC, which is dependent on GPR40-mediated Ca$^{2+}$ release (59). The experiments reported here are not able to address the temporal sequence of this two-component rise of $[\text{Ca}^{2+}]_i$ in the native system. However, because COS-7 and HEK-293 do not express GPR40, this clearly rules out a requirement for GPR40 activation in oleate-induced potentiation of LTCC Ca$^{2+}$ currents, even if the precise mechanism(s) remains to be clarified. The potentiation that we observed did not appear to require calcium influx, because a similar degree of potentiation was observed when calcium was replaced by barium in the extracellular solution. This work also does not elucidate whether the Ca$^{2+}$ channel itself or another protein in intimate association with the channel is the direct target of fatty acids or their
LC-CoA derivatives. Our use of triacsin C, which as a specific inhibitor of acyl-CoA synthetases blocks the first step in intracellular utilization of fatty acids, suggests that the oleoyl-CoA and not oleate itself may be required for potentiation to occur.

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OLEATE MODULATION OF CLONED Ca CHANNELS


