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Ca^{2+} binding protein-1 inhibits Ca^{2+} currents and exocytosis in bovine chromaffin cells

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Summary

Calcium binding protein-1 (CaBP1) is a calmodulin like protein shown to modulate Ca^{2+} channel activities. Here, we explored the functions of long and short spliced CaBP1 variants (L- and S-CaBP1) in modulating stimulus-secretion coupling in primary cultured bovine chromaffin cells. L- and S-CaBP1 were cloned from rat brain and fused with yellow fluorescent protein at the C-terminal. When expressed in chromaffin cells, wild-type L- and S-CaBP1s could be found in the cytosol, plasma membrane and a perinuclear region; in contrast, the myristoylation-deficient mutants were not found in the membrane. More than 20 and 70% of Na^+ and Ca^{2+} currents, respectively, were inhibited by wild-type isoforms but not myristoylation-deficient mutants. The $[Ca^{2+}]_i$ response evoked by high K^+ buffer and the exocytosis elicited by membrane depolarizations were inhibited only by wild-type isoforms. Neuronal Ca^{2+} sensor-1 and CaBP5, both are calmodulin-like proteins, did not affect Na^+, Ca^{2+} currents, and exocytosis. When expressed in cultured cortical neurons, the $[Ca^{2+}]_i$ responses elicited by high-K^+ depolarization were inhibited by CaBP1 isoforms. In HEK293T cells cotransfected with N-type Ca^{2+} channel and L-CaBP1, the current was reduced and activation curve was shifted positively. These results demonstrate the importance of CaBP1s in modulating the stimulus-secretion coupling in excitable cells.

Abbreviations: CaBP – calcium binding protein; CaM – calmodulin; I_{Ca} – Ca^{2+} currents; I_{Na} – Na^+ currents; IP_3 – inositol 1,4,5-trisphosphate; NCS-1 – neuronal calcium sensor-1

Introduction

Calcium homeostasis is very important in many physiological events such as neurotransmitter release, muscle contraction, and gene expression [1, 2]. Therefore, the mechanism by which the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is modulated is an important issue for cellular activities. The main sources of $[Ca^{2+}]_i$ elevation are from extracellular solution and intracellular Ca^{2+} stores. The voltage-gated Ca^{2+} channel in the plasma membrane is the major pathway to allow Ca^{2+} influx into the cytosol; inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors are responsible for Ca^{2+} release from intracellular Ca^{2+} stores. These pathways contribute differentially to $[Ca^{2+}]_i$ elevation depending on the characteristics of the stimulations involved [3–5].

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The change in the amount of freely available cytosolic Ca^{2+} is vital to the activities of many proteins, which typically have the ability to bind Ca^{2+} [6, 7]. Calmodulin (CaM) has 4 EF-hand Ca^{2+}-binding motifs; the binding of Ca^{2+} induces a conformational change and converts CaM into an active form able to modulate many physiological functions [6, 8]. Recently, a group of calcium binding proteins (CaBP1$\sim$5) with structures similar to CaM has been described [9]. These CaBPs may have different subcellular localizations for sensing the changes in local $[Ca^{2+}]_i$ homeostasis to differentially modulate cellular activities. CaBP1 has been shown to directly activate the IP_3 receptors [10]; however, it has also been suggested to inhibit the elevation in $[Ca^{2+}]_i$ induced by ATP, which opens the IP_3 receptors, in PC12 cells [11].

CaBP1 differs from CaM in that it has an N-terminal myristoylation moiety and an inactive EF-2. It has two alternative spliced variants, L- and S-CaBP1 [12]. L-CaBP1 has been extensively studied for its effects on various types of Ca^{2+} channels. It binds to the C-terminal CaM binding domain of $Ca_v2.1$ and enhances current inactivation [13]; when binding to the IQ domain of $Ca_v1.2$, it prolongs Ca^{2+} currents (I_{Ca}) [14, 15]. These findings suggest that L-CaBP1 differentially modulates the activities of various Ca^{2+} channels. However, the roles of L- and S-CaBP1 in modulating Ca^{2+} currents and neurotransmitter release in excitable cells have not been reported.

Here we investigate the physiological roles of L- and S-CaBP1s in modulating Ca^{2+} channels and neurotransmitter release in excitable cells. L- and S-CaBP1 cloned from freshly isolated rat E14.5 cortex were transiently expressed in bovine chromaffin cells and cortical neurons. To our knowledge this is the first report to show that both L- and S-CaBP1 have similar effects in attenuating stimulus-secretion coupling by diminishing I_{Ca}. Our results reveal the importance of CaBP1 in modulating the Ca^{2+} signaling and neurotransmitter release in excitable cells.

Methods

Chemicals

Fura-2 acetomethoxylmethyl ester (Fura-2 AM) was purchased from TefLabs (Austin, TX, USA). Ca^{2+}, Mg^{2+}-free Hank's Balanced Salt Solution, Neurobasal Medium, B27, DMEM and other chemicals for cell culture were from Invitrogen Inc. (Carlsbad, CA, USA). All other chemicals were commercially available and of reagent grade from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise indicated.

Solutions

The normal bath buffer used for calcium imaging and electrophysiological recording contained (in mM): 145 NaCl, 5 glucose, 10 Na-HEPES, 1 $MgCl_2$, 5 KCl, and 2.2 $CaCl_2$, pH 7.3 with NaOH. To record the N-type I_{Ca} expressed in human embryonic kidney 293 T (HEK293T) cells, NMG buffer (in mM, 140 *N*-methyl-D-glucamine (NMG), 5 glucose, 10 Na-HEPES, 1 $MgCl_2$, and 10 $CaCl_2$, pH7.3 with NaOH) was used as the bath solution. To depolarize the cells, high K^+ buffer (in mM): 150 KCl, 5 glucose, 10 Na-HEPES, 1 $MgCl_2$, and 2.2 $CaCl_2$, pH 7.3 with KOH, was used to perfuse the cells locally. The Cs^+-containing pipette solution for electrophysiological recording contained (in mM): 120 Cs-aspartate, 40 Na-HEPES, 5 $MgCl_2$, 0.1 EGTA, 2 ATP, 0.3 GTP, pH7.3 with CsOH. 2X BES-buffered saline contained (in mM): 50 N-bis[2-hydroxyethyl] 2-aminoethanesulfonic acid (BES), 273 NaCl, and 2.2 NaH_2PO_4, pH 6.96.

Isolation and culture of rat E14.5 cortical neuron

E14.5 embryos were obtained from pregnant Sprague-Dawley rat by Caesarian section complying with the regulations of animal welfare regulation of the National Taiwan University. The forebrain was isolated under dissecting microscope and digested with papain (1 mg/ml in Ca^{2+}, Mg^{2+}-free HBSS) at 37 °C with gentle shaking for 20 min. The tissue was triturated by 1 ml glass pipette for 20 strokes and centrifuged at $300 \times g$ for 1 min. The pellet was resuspended in Ca^{2+}, Mg^{2+}-free HBSS and adjusted to a density of 10^6 cells/ml. Isolated cells were cultured according to published protocol [16]. To inhibit the growth of glia cells, cytosine arabinoside (1 μg/ml) was added in the culture medium.

Isolation and culture of bovine chromaffin cells

Chromaffin cells were prepared by digestion of bovine adrenal gland obtained from local slaugh-

terhouses with collagenase (0.5 mg/ml) and purified by density gradient centrifugation as previously described [17]. In brief, cells were plated at a density of 2×10^5 cells per 35-mm culture dish on poly L-lysine-coated coverslips and cultured in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum. The medium was replaced every two days. All experiments were carried out between 5 and 10 days after cells were isolated.

Culture of HEK293T cells

HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum in a humidified incubator under 5% CO_2.

Molecular cloning

The total RNA of isolated rat embryonic cortex or cultured bovine chromaffin cells was collected using Trizol reagent (Invitrogen Inc., USA) according to the manufacturer's manual. Single strand cDNA was synthesized from the total RNA using oligo-dT as the primer by Superscriptase III (Invitrogen Inc., USA). Specific primer sets with linkers (underlined) containing restriction enzyme cutting sites (in parenthesis) were designed to clone these genes from rat brain cDNA: (*Hin*dIII) 5′-GCCG(AAGCTT)ATGGGCAACTGCGTCAAGTC-3′ and (*Sac*II) 5′-GACG(CCGCGG)GCGAGACATCATCCGGACAA-3′ for L- and S-CaBP1; (*Hin*dIII) 5′-GCCG(AAGCTT)ATGGCCAACTGCGTCAAGTC-3′ and (*Sac*II) 5′-GACG(CCGCGG)GCGAGACATCATCCGGACAA-3′ for L- and S-CaBP1/G2A; (*Xho*I) 5′- ATA(CTCGAG)ATGCAGTTTCCCATGGGCC-3′ and (*Bam*HI) 5′-ATTA(GGATCC)AACGAGACATCATCTTCACA-3′ for CaBP5; (*Xho*I) 5′-CAT(CTCGAG)ATGGGGAAATCCAACAGCAAGT-3′ and 5′ (*Bam*HI) CAT(GGATCC)AATACCAGCCCGTCGTAGAGGG-3′ for NCS-1. The PCR products were subcloned into a pEYFP-N1 plasmid (Clontech Laboratories Inc., Mountain View, CA, U. S. A.) and verified by DNA sequencing. N-type Ca^{2+} channel and the accessory subunits are a generous gift from Dr. Aaron Fox [18].

Transfection

To transiently express those constructs in chromaffin, neuronal, and 293 T cells, calcium phosphate transfection was used, as described previously [19]. In brief, plasmids (5 μg) were mixed with 25 μl 1 M $CaCl_2$, 50 μl 2X BES-buffered saline and water to make a final volume of 100 μl for 30 min. This mixture was added into a 35-mm cultured dish which contained cells and 1 ml of DMEM for 1 h. Cells were then shocked by 600 μl of 10 % glycerol in DMEM for 1 min (for chromaffin cells only). The glycerol solution was immediately washed out and DMEM containing 10% fetal bovine serum was added. The transfected cells were incubated in a humidified CO_2 (5%) incubator and used in 2 days.

Fluorescence Imaging

Transfected cells were fixed with 3.7% of formaldehyde in bath buffer for 1 h and permeablized by Triton X-100 buffer (0.1% Triton X-100 in phosphate buffered saline, PBS) for 10 min. Cells were then incubated in blocking buffer (1% BSA in PBS) for 1 h and stained with 2 units of rhodamine-conjugated phalloidin (Invitrogen Inc., USA) for 30 min as described by the manufactured manual. Hoechst 33258 (Invitrogen Inc., USA) was added into the solution 1 min before the end of staining. The stained cells were mounted on the Leica DM IRE2 epifluorescence microscope and observed by a $100 \times$ oil objective, N.A. 1.45. Fluorescence images were taken by Retiga Exi CCD camera (Qimaging Inc., BC, Canada) with appropriate band pass filter sets. The fluorescence intensity and images were analyzed by the deconvolution module in Simple PCI Software (Compix Inc., Sewickley, PA, USA).

Calcium imaging

For $[Ca^{2+}]_i$ measurement, cells were incubated in normal bath buffer with 5 μM fura-2 AM for 1 h at 37 °C. Cells were then washed 3 times with normal bath buffer and used for measurements as described before [20]. The ratio within each cell was computed from images obtained at 340 and 380 nm excitation wavelengths and subtracting the appropriate background fluorescence at each wavelength. Single transfected cells were stimu-

lated with high K^+ buffer puffed from a micropipette and the ratio was acquired every second. The ratios before stimulation were averaged as the basal and subtracted from the ratios measured after stimulation to obtain the maximal changes in fura-2 responses.

Electrophysiological measurements

Cells were transferred to a recording chamber mounted on the stage of an inverted microscope and incubated in bath buffer at room temperature (25–28 °C). Patch pipettes were pulled from thin-wall capillaries with filament (Catalog 617000, A-M Systems Inc., Everett, WA, USA) using a two-stage microelectrode puller (P-97, Sutter Instrument, Novato, CA, USA), and fire-polished with a microforge (MF-830, Narishige Inc., Japan). When filled with pipette solution, the resistance ranged between 3 and 5 MΩ. To monitor the change in membrane capacitance, electrodes were coated with Sylgard (Catalog 184 Silicone Elastomer Kit, Dow Corning Co., Midland, MI, USA) to reduce nonspecific noise. Ionic currents and membrane capacitance were measured from whole-cell patch-clamped cells using an EPC10 patch-clamp amplifier (HEKA GmbH, Germany) and controlled by Pulse software (HEKA GmbH, Lambrecht, Germany).

For capacitance measurements, cells were whole-cell voltage clamped at −70 mV and depolarized with a train of 10 depolarizations to +10 mV for 150 ms with an interval of 150 ms between the start of two consecutive depolarizations. A 10-ms sinewave with a frequency of 1 kHz and amplitude of 20 mV was applied just before the start of each depolarization to monitor the membrane capacitance. After the end of this train of depolarizations, the same sinewave was applied continuously and the capacitance measured was averaged every 100 ms. The membrane capacitance was obtained by the Lock-in amplifier using sine + dc mode in the Pulse software program.

To monitor the whole-cell inward I_{Na} and I_{Ca} in chromaffin cells, cells were voltage-clamped at a holding potential of −70 mV and depolarized to various potentials for 30 ms once every 15 s. The maximal inward current obtained during the first 5 ms was identified as I_{Na} and the current recorded between 18 and 27 ms of depolarization as I_{Ca}. To measure the N-type I_{Ca} expressed in HEK293T cells, cells were voltage-clamped at a holding potential of −90 mV and depolarized to various potentials for 200 ms once every 10 s. The maximal currents obtained during the depolarizations and right after depolarizations were recorded for current–voltage relationship and activation curve, respectively.

Signals were low-pass filtered at 3 kHz and stored in a Pentium III-based computer. Data are presented as mean ± S.E.M. Unpaired Student's *t*-test was used for statistical evaluation of differences among means. A value of $p < 0.05$ was considered to be statistically significant. Normalized curves were fit to a Boltzmann function, using the least-squares method according to: $A/\{1 + \exp[(V - V_h)/\text{slope}] + b\}$, where V is the test voltage, V_h is the midpoint of the activation curve, A is the amplitude and b is the baseline.

Results

Subcellular localization of CaBP1s

To identify the subcellular localization of the CaBP1s, constructs carrying fused YFP to the C-terminal of L- and S-CaBP1s were transiently expressed in bovine chromaffin cells and stained with Hoechst 33258 and rhodamine-conjugated phalloidin to label the nucleus and F-actin, respectively (Figure 1). YFP in cells transfected with empty vector was found to be mainly localized in the nucleus and partly in the cytosol. F-actin has been suggested to be found right under the plasmalemma region in chromaffin cells [21]. L-CaBP1 has been shown to present on the plasma membrane and the perinucler region in PC12 cells [11]; similarly, L- and S-CaBP1 were mainly found in the plasma membrane and perinuclear region. In contrast, the myristoylation-defective G2A mutants were localized in the nucleus and perinuclucler region. These findings indicate the importance of myristoylation in the anchoring of the L- and S-CaBP1 to the plasma membrane.

L- and S-CaBP1 inhibit $[Ca^{2+}]_i$ responses stimulated by high K^+ buffer

L-CaBP1 has been shown to modulate L- and P/Q-type Ca^{2+} channels [15, 22]. It is not clear

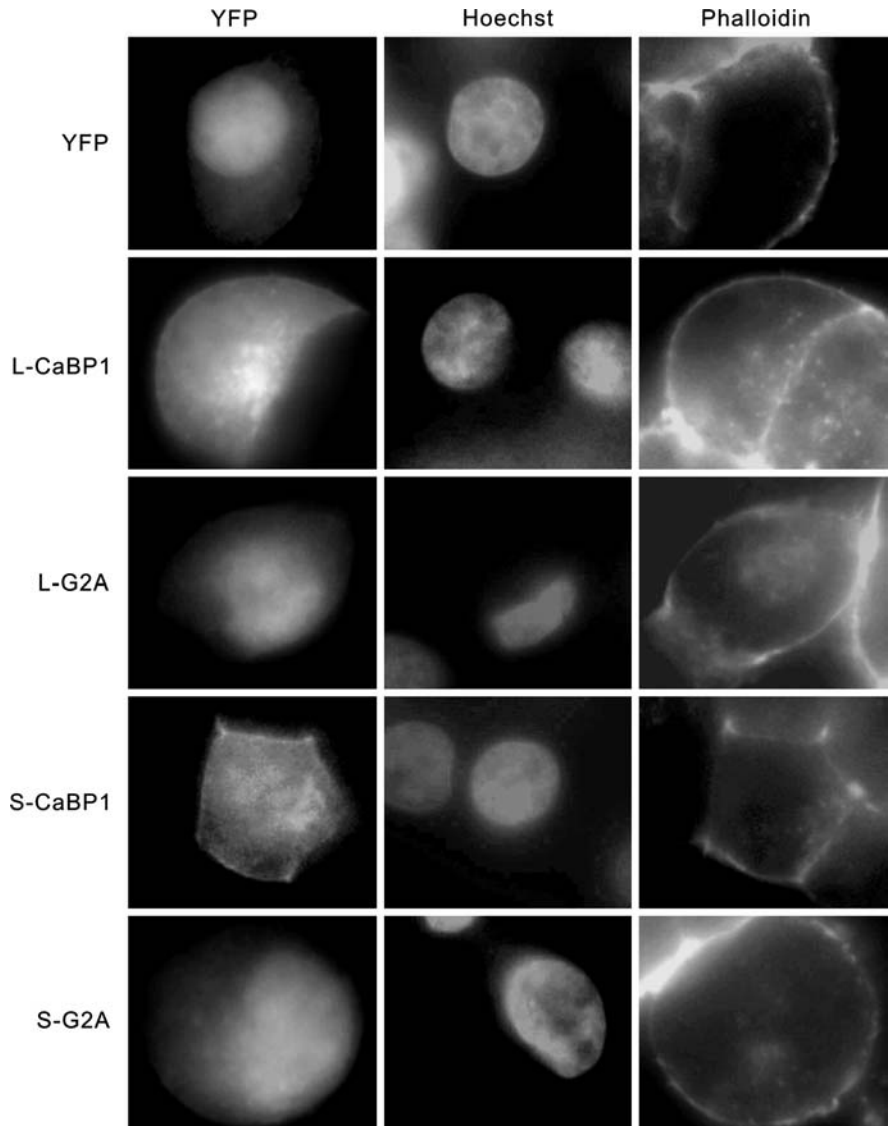


Figure 1. Deconvoluted images of exogenously expressed CaBP1s in bovine chromaffin cells. Chromaffin cells expressing YFP, and YFP fusion proteins of L-CaBP1, L-CaBP1/G2A (L-G2A), S-CaBP1, and S-CaBP1/G2A (S-G2A) (from the top to bottom rows) were fixed with formaldehyde and stained with Hoechst 33258 (Hoechst, middle column) and rhodamine-conjugated phalloidin (Phalloidin, right column) to label the nucleus and F-actin, respectively. The YFP fluorescence images of each construct are displayed in the left column. The images were observed under a Leica epifluorescence microscope (100×, NA1.45, oil objective) equipped with a CCD camera and analyzed by software-deconvolution. The black bar indicates 5 μm.

whether S-CaBP1 also has such an effect on Ca^{2+} signaling, or how L- and S-CaBP1 modulate $[Ca^{2+}]_i$ responses in excitable cells. To address these questions, chromaffin cells transfected with L- and S-CaBP1 were stimulated by high K^+ buffer (150 mM) for 1 s and the changes in $[Ca^{2+}]_i$ were monitored by the fura-2 fluorescence ratio. To minimize the non-specific effects caused by overexpression, cells with dim YFP signals were selected by eye under microscope for experiments. The representative results (Figure 2a) show that the ratio was quickly elevated in cell expressing YFP; this elevation was diminished in cells expressing L- or S-CaBP1. In contrast, in cells expressing L- and S-CaBP1/G2A mutants, the elevations in the fura-2 ratio elicited by high

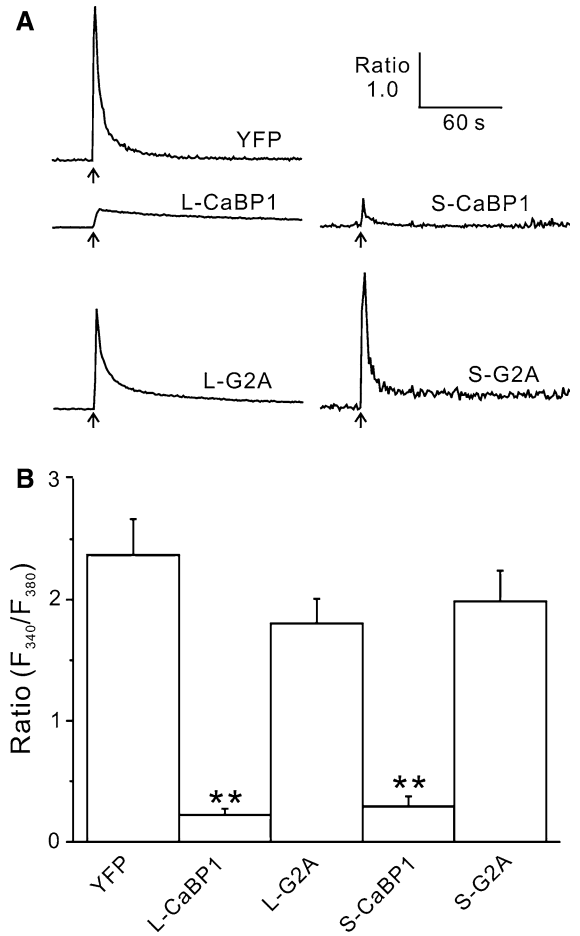
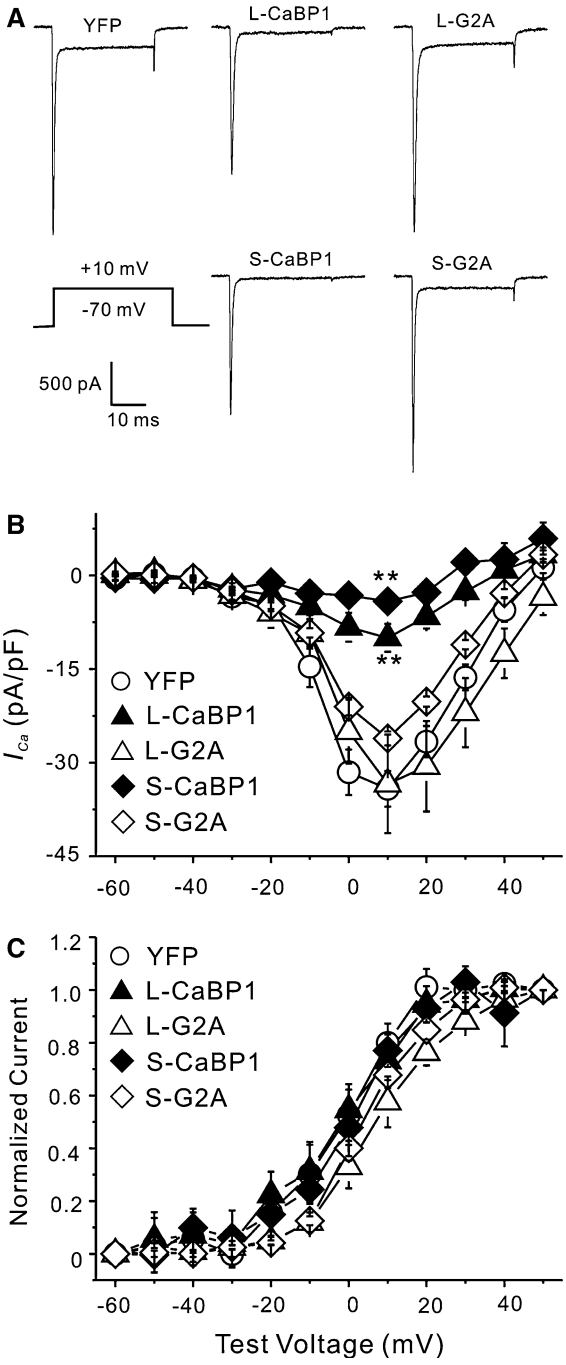


Figure 2. $[Ca^{2+}]_i$ response in transfected chromaffin cells. Single transfected chromaffin cells were stimulated with high K^+ buffer (contained 150 mM KCl) for 1 s (as indicated by the arrow) and the changes in $[Ca^{2+}]_i$ were monitored changes in the fura-2 fluorescence ratio. (a) Representative $[Ca^{2+}]_i$ responses from single chromaffin cells expressing YFP, L-CaBP1, L-CaBP1/G2A (L-G2A), S-CaBP1, S-CaBP1/G2A (S-G2A). (b) Averaged changes in $[Ca^{2+}]_i$. The elevations in $[Ca^{2+}]_i$ after basal subtraction were averaged. Data are the mean ± S.E.M. from at least 10 cells for each group; **: Student's *t*-test at $p < 0.01$ when compared to cells expressing YFP.

K^+ buffer were similar to that expressing YFP. The averaged results (Figure 2b) show that the change in the fura-2 ratio was 2.4 ± 0.3 in cells expressing YFP; this response was significantly reduced by the expression of L- and S-CaBP1 to 0.2 ± 0.0 and 0.3 ± 0.1, respectively. In contrast, L- and S-CaBP1/G2A slightly but not significantly reduced the ratio to 1.8 ± 0.2 and 2.0 ± 0.3, respectively. These results indicate that L- and S-CaBP1 block the $[Ca^{2+}]_i$ response stimulated by membrane depolarization.



L- and S-CaBP1 inhibit Ca^{2+} currents

To characterize the effects of L- and S-CaBP1s on inward currents, chromaffin cells were transfected with YFP-fused L- and S-CaBP1 and whole-cell patched (Figure 3). The peak inward current (due

Figure 3. Effects of CaBP1s on Ca^{2+} currents. Cells were bathed in normal bath buffer and whole-cell voltage-clamped using a Cs^{+}-containing pipette solution. Depolarizations (30 ms) to various potentials were applied once every 15 s from a holding potential of −70 mV. The inward maximal peak currents was recorded as the Na^{+} current (I_{Na}); the current between the 18th and 27th ms of the depolarization was averaged and recorded as the Ca^{2+} current (I_{Ca}). (a) Representative current traces during depolarizations to +10 mV from cells expressing YFP, L-CaBP1, L-CaBP1/G2A (L-G2A), S-CaBP1, or S-CaBP1/G2A (S-G2A). (b) Averaged current-voltage and (c) Normalized Tail Current-voltage relations of I_{Ca} from cells expressing YFP (○), L-CaBP1 (▲), L-CaBP1/G2A (L-G2A) (Δ), S-CaBP1 (◆), or S-CaBP1/G2A (S-G2A) (◇). Data are the mean ± S.E.M. from at least 6 cells each; **Student's *t*-test at $p < 0.01$ when compared to cells expressing YFP.

largely to I_{Na}) and sustained inward current (due largely to I_{Ca}) obtained from representative transfected cells were both inhibited by the expression of L- and S-CaBP1, but not their G2A mutants, using depolarizations from a holding potential of −70 mV to +10 mV (Figure 3a). The averaged current densities of I_{Ca} (Figure 3b) obtained from cells expressing YFP was −34.2 ± 2.9 pA/pF at +10 mV; this was significantly ($p < 0.01$) reduced by the expression of L- and S-CaBP1 to −10.0 ± 2.2 and −4.2 ± 1.1, respectively. For cells expressing myristoylation-deficient G2A mutants, this inhibition was not significant, i.e. −33.4 ± 7.9 and −26.1 ± 1.1 pA/pF for L- and S-CaBP1/G2A, respectively. The normalized tail current–voltage curves (Figure 3c) were not significantly shifted by these constructs. The V_h and slope factor for chromaffin cells expressing YFP, L-CaBP1, L-CaBP1/G2A, S-CaBP1, and S-CaBP1/G2A were −1.0 ± 1.1 and 8.6 ± 1.0; −1.0 ± 1.4 and 10.9 ± 1.3; 7.7 ± 0.7 and 9.9 ± 0.6; 0.5 ± 1.4 and 8.3 ± 1.3; 4.2 ± 0.5 and 8.3 ± 0.5 mV, respectively. These results suggest that both L- and S-CaBP1 inhibit the inward I_{Ca}, and the N-terminal myristoylation modification is required for this inhibition.

L- and S-CaBP1 inhibit Na^{+} currents

To examine the effects of CaBP1s on I_{Na}, the peak inward currents obtained in the previous figure were analyzed and plotted against the depolarization potentials. The averaged densities of I_{Na} (Figure 4a) at 0 mV were significantly reduced from −309.3 ± 20.4 pA/pF, in cells expressing

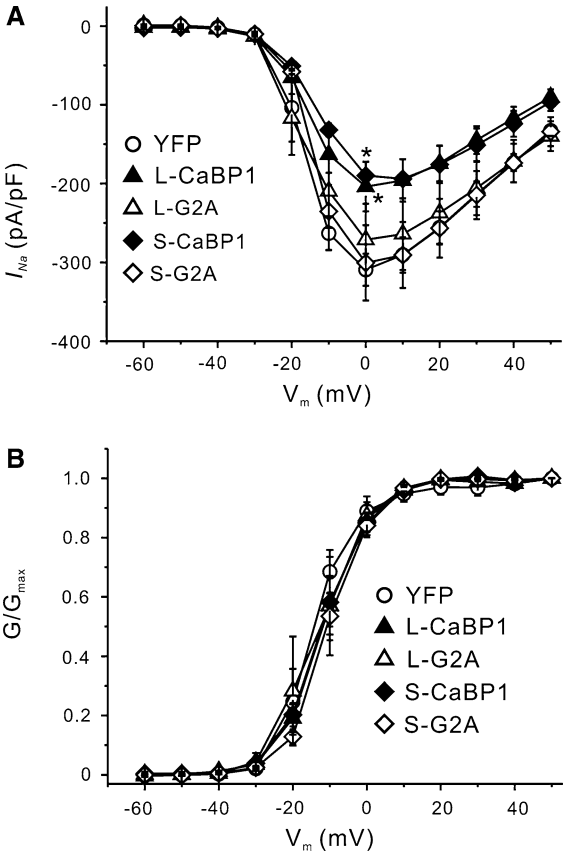


Figure 4. Effects of CaBP1s on Na^{+} currents. Transfected chromaffin cells were whole-cell voltage-clamped and the inward currents were recorded as described in the previous figure. (a) Average *I–V* relations of I_{Na} from cells expressing YFP (○), L-CaBP1 (▲), L-CaBP1/G2A (L-G2A) (Δ), S-CaBP1 (◆), and S-CaBP1/G2A (S-G2A) (◇). (b) Voltage dependence of activation. Currents obtained at different potentials were normalized to the maximal conductance. Data are the mean ± S.E.M. from at least 6 cells each; *: Student's *t*-test at $p < 0.05$ when compared to cells expressing YFP.

YFP, to −204.0 ± 31.4 and −190.0 ± 17.8, by the expression of L- and S-CaBP1, respectively. For cells expressing G2A mutants, this inhibition was not significant, i.e. −281.2 ± 45.5 and −300.5 ± 47.7 pA/pF for L- and S-CaBP1/G2A, respectively. When normalized to the maxima conductance (Figure 4b), there was no significant difference among these curves in V_h and slope factor when fitted by Boltzmann function. These results suggest that both L- and S-CaBP1 inhibit the amplitude of I_{Na}, but have no significant effect on the voltage-dependent activation curve.

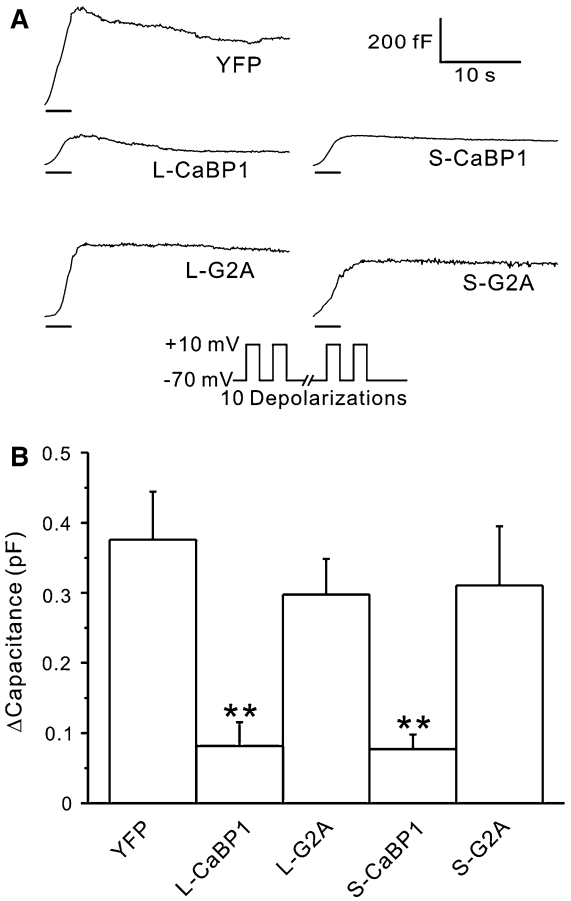


Figure 5. Effects of CaBP1s on depolarization-evoked exocytosis. Cells were placed in normal bath buffer and whole-cell patched with a Cs^+-containing pipette solution. Exocytosis was evoked by a train of 10 depolarizations to +10 mV from a holding potential of −70 mV for 150 ms with an interval of 150 ms. The membrane capacitance during and after the depolarizations was recorded. The total recording period including the train of depolarizations was 40 s. (a) Representative capacitance traces from cells transfected with YFP, L-CaBP1, L-CaBP1/G2A (L-G2A), S-CaBP1, or S-CaBP1/G2A (S-G2A). The black bar under each trace indicates the period of 10 depolarizations. (b) Averaged maximal changes in capacitance after baseline subtraction during the recording period. Data are the mean ± S.E.M. from at least 6 cells each; **: Student's *t*-test at $p < 0.01$ when compared to cells expressing YFP.

L- and S-CaBP1 inhibit exocytosis

$[Ca^{2+}]_i$ elevation is a prerequisite for evoked exocytosis [1]. To investigate whether the inhibition of I_{Ca} by CaBP1s leads to the modulation of exocytosis, the changes in membrane capacitance evoked by a train of depolarizations were monitored (Figure 5). When exocytosis is evoked by membrane depolarization, the fusion of the secretory vesicles to the plasma membrane increases the surface area, which is reflected in an increase in membrane capacitance. In a control cell expressing YFP, a train of 10 depolarizations resulted in a rapid increase in membrane capacitance; in cells expressing L- or S-CaBP1, the increase was small. The averaged increase in capacitance during the train of depolarizations was 0.38 ± 0.07 pF for cells expressing YFP; this was significantly diminished to 0.08 ± 0.03 and 0.08 ± 0.02 by L- and S-CaBP1, respectively. In contrast, the depolarization-evoked exocytosis was not reduced by G2A mutants, being 0.29 ± 0.05 and 0.31 ± 0.08 pF for L- and S-CaBP1/G2A, respectively. These results indicate that L- and S-CaBP1 attenuate stimulus-secretion coupling in chromaffin cells.

CaBP5 and NCS-1 do not affect the exocytosis and inward currents

CaBP5 is homologous to CaBP1 but without the N-terminal myristoylation conserved sequence; it was localized in the cytosol (data not shown). NCS-1 is another CaM-like protein with an N-terminal myristoylation modification and has been shown to be present in the plasma membrane [12, 23]. Both were cloned from rat embryonic cortical neurons and fused with YFP at the C-terminus. Chromaffin cells were transfected with CaBP5 and NCS-1 to characterize their effects on ionic currents and exocytosis (Table 1). The results show that these two proteins did not have any

Table 1. Normalized effects of L-CaBP1, S-CaBP1, NCS-1, and CaBP5 on stimulus-secretion coupling in chromaffin cells.

	Exocytosis (%)[a]	I_{Ca} (%)[b]	I_{Na} (%)[b]
L-CaBP1	21.7 ± 9.0	29.3 ± 6.5	67.5 ± 9.2
S-CaBP1	20.5 ± 5.5	12.2 ± 3.3	66.8 ± 8.5
NCS-1	86.6 ± 18.5	108.3 ± 16.4	95.6 ± 14.5
CaBP5	104.2 ± 14.3	99.6 ± 14.9	111.5 ± 16.3

[a]The maximal exocytosis evoked by a train of 10 depolarizations was averaged and normalized to that of cells expressing YFP.
[b]I_{Ca} and I_{Na} obtained by a step depolarization to +10 mV from a holding potential of −70 mV for 30 ms were averaged and normalized to that of cells expressing YFP.

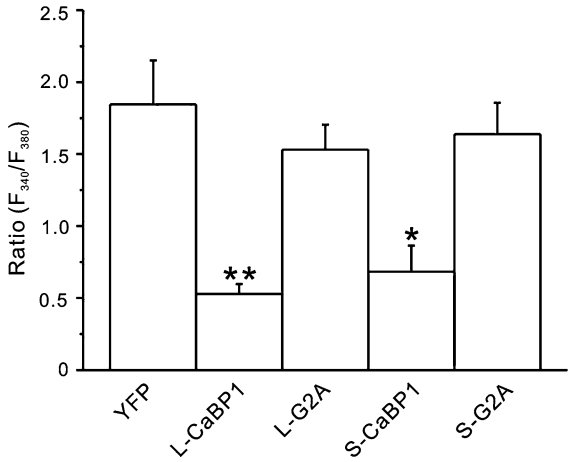


Figure 6. $[Ca^{2+}]_i$ response in cortical neurons expressing CaBP1s. Transfected cortical neurons were placed in normal bath buffer and stimulated with high K^+ buffer (containing 150 mM K^+) for 1 s and the $[Ca^{2+}]_i$ was monitored as the change in the fura-2 fluorescence ratio. The changes in $[Ca^{2+}]_i$ from neurons expressing YFP, L-CaBP1, L-CaBP1/G2A (L-G2A), S-CaBP1, or S-CaBP1/G2A (S-G2A) were averaged after basal subtraction. Data are the mean ± S.E.M. from at least 10 cells each; * and **: Student's *t*-test at $p < 0.05$ and 0.01, respectively, when compared to cells expressing YFP.

significant effects on I_{Na}, I_{Ca}, or exocytosis. These results illustrate that different CaM-like proteins have differential effects in modulating the stimulus-secretion coupling of excitable cells and exclude the possibility that the inhibitory effect of CaBP1 is a non-specific result due to the overexpression of CaBPs.

L- and S-CaBP1 inhibit the high K^+-induced $[Ca^{2+}]_i$ responses in neurons

To understand whether L- and S-CaBP1 in neurons have similar effects in modulating Ca^{2+} signaling, the elevations in $[Ca^{2+}]_i$ elicited by high K^+ buffer in cultured embryonic cortical neurons were examined (Figure 6). The averaged elevation in $[Ca^{2+}]_i$ was 1.8 ± 0.3 for neurons expressing only YFP and significantly diminished to 0.5 ± 0.1 and 0.7 ± 0.2 by the expression of L- and S-CaBP1, respectively. In contrast, the elevations in $[Ca^{2+}]_i$ were not reduced by G2A mutants, at 1.5 ± 0.2 and 1.6 ± 0.2 for L- and S-CaBP1/G2A, respectively. These results suggest that L- and S-CaBP1s may have a similar inhibitory effect on Ca^{2+} signaling in neurons.

L-CaBP1 inhibits N-type Ca^{2+} current

To characterize whether N-type Ca^{2+} channels, which constitutes 50% of the total I_{Ca} in the chromaffin cells isolated in our lab (unpublished result), are modulated by CaBP1; N-type Ca^{2+} channel cloned from bovine chromaffin cells [18] was co-expressed with L-CaBP1 in HEK293T cells. Transfected cells were incubated in NMG buffer containing 10 mM $CaCl_2$ and whole-cell patched with a Cs^+-containing pipette solution. Cells were voltage-clamped at -90 mV and depolarized to various potentials for 200 ms once every 10 s (Figure 7). The representative current traces show that, when depolarized to +30 mV, an inward current could be elicited from a cell cotransfected with YFP and N-type Ca^{2+} channel; this current was reduced in a cell expressing L-CaBP1 but not L-CaBP1/G2A. The averaged current density (Figure 7b) obtained from cells cotransfected with YFP and Ca^{2+} channel was −13.4 ± 1.9 pA/pF when depolarized to +30 mV; this was significantly ($p < 0.05$) reduced by the expression of L-CaBP1 to −3.2 ± 1.4 pA/pF. For cells expressing myristoylation-deficient L-CaBP1/G2A mutant, this inhibition was not significant, i.e. −12.6 ± 4.1 pA/pF. In cells expressing N-type Ca^{2+} currents, the normalized tail current-voltage curve (Figure 7c) was positively shifted by the L-CaBP1 comparing to cells expressing YFP. L-CaBP1 caused significant changes in the V_h (44.9 ± 2.8 mV for L-CaBP1 versus 31.9 ± 0.9 mV for YFP, $p < 0.05$), and slope factor (17.5 ± 1.8 mV for L-CaBP1 versus 12.1 ± 0.9 mV for YFP, $p < 0.05$). G2A mutant did not have such effect on the activation curve (V_h and slope factor were 31.5 ± 1.0 and 12.2 ± 0.9 mV, respectively). These results demonstrate that L-CaBP1 inhibits the N-type Ca^{2+} currents and affecting the activation curve.

Discussion

The results in this report show that both L- and S-CaBP1 inhibit I_{Ca} and I_{Na} in bovine chromaffin cells. The exocytosis and $[Ca^{2+}]_i$ response evoked by depolarization were blocked by the overexpression of L- and S-CaBP1. Without the N-terminal myristoylation modification, these isoforms could not localize to the plasma membrane to modulate

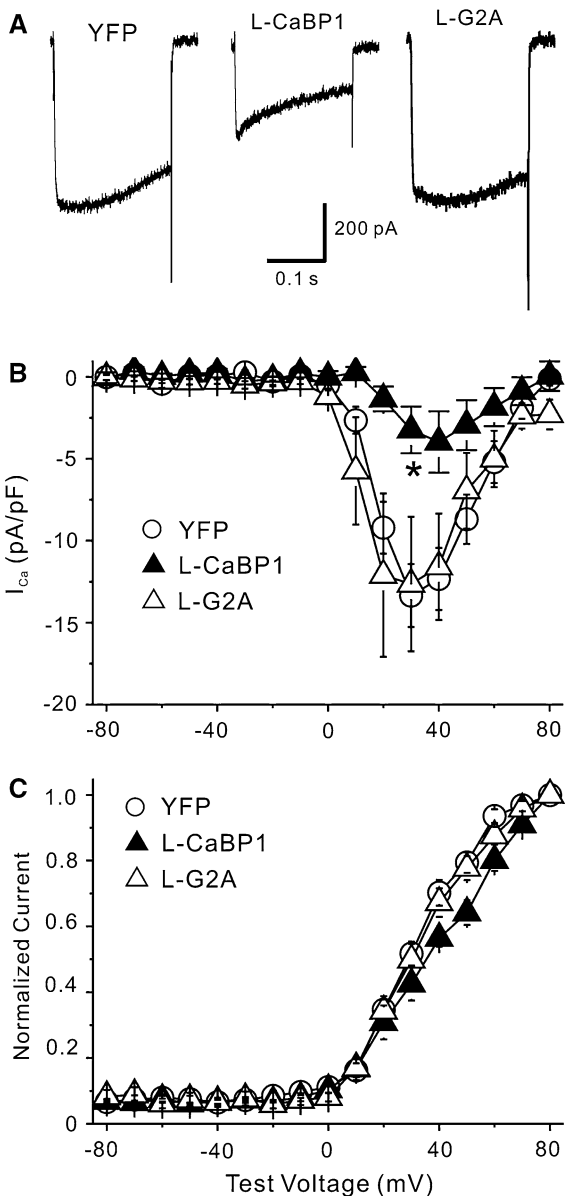


Figure 7. L-CaBP1 inhibits N-type Ca^{2+} currents. HEK293T cells cotransfected with L-CaBP1 and N-type Ca^{2+} channels were incubated in NMG buffer and patched with a Cs^{+}-containing pipette solution. Depolarizations (200 ms) to various potentials were applied once every 10 s from a holding potential of −90 mV. (a) Representative current traces during depolarizations to +30 mV from cells expressing YFP, L-CaBP1, and L-CaBP1/G2A (L-G2A). (b) Averaged Current-Voltage and (c) Normalized Tail Current-voltage relations of I_{Ca} from cells expressing YFP (○, $n = 24$), L-CaBP1 (▲, $n = 7$), and L-CaBP1/G2A (L-G2A) (Δ, $n = 9$). Data are the mean ± S.E.M. *: Student's t-test at $p < 0.05$ when compared to cells expressing YFP.

the ion channel activities. When expressed in cultured neurons, the $[Ca^{2+}]_i$ response elicited by high K^{+} buffer was also inhibited by L- and S-CaBP1. In HEK293T cells contransfected with L-CaBP1 and N-type Ca^{2+} channel, the currents were inhibited. These findings demonstrate the importance of L- and S-CaBP1 in attenuating stimulus-secretion coupling in excitable cells.

When expressed in chromaffin cells, YFP is localized in the nucleus; however, this did not interfere with the localization of CaBP1-YFP fusion proteins to the plasma membrane. In PC12 cells, the localization of L-CaBP1 is in the plasma membrane and a perinuclear region which is probably the Golgi complex [11]. The reason L- and S-CaBP1, even without myristoylation modification, are concentrated in this perinuclear region needs to be further investigated.

Overexpression of YFP in bovine chromaffin cells significantly attenuated both the I_{Na} and I_{Ca} by 10 and 25%, respectively (unpublished results); similar findings have been reported before [24, 25]. However, the expression of CaBP1s further inhibits the I_{Na} and I_{Ca} by more than 35 and 70% of that from cells expressing YFP, respectively. In contrast, myristoylation-deficient mutants and two other CaM-like proteins, NCS-1 and CaBP5, did not have such significant inhibitory effects. These results reveal that inhibitory effect on I_{Ca} and I_{Na} is a specific characteristic of CaBP1s which results in attenuating the stimulus-secretion coupling.

The results with the myristoylation-deficient mutants, L- and S-CaBP1/G2A, show that this modification is required for modulating the inward currents and exocytosis. It is unlikely that the inability of the G2A mutants to modulate I_{Ca} is due to lower expression levels in transfected cells as the fluorescence intensity in those cells used for recording are about the same (data not shown). In addition, NCS-1 has the N-terminal myristoylation modification and has been shown to localize in the plasma membrane [25, 26]; CaBP5 has a high homology with CaBP1 but without the myristoylation consensus sequence [9] and is localized in the cytosol when expressed in cortical neuron (data not shown). Both have no effect on the inward currents, excluding the possibility that the inhibition on ionic currents

and exocytosis is a non-specific effect due to the overexpression of these CaM-like proteins. L-CaBP1/G2A has been shown to be able to associate with $Ca_v2.1$, but has no effect on the channel properties [27]. These findings indicate that localization to the plasma membrane is required but not the only factor responsible for the modulation of the current.

CaBP5 could be cloned from freshly isolated embryonic cortical neurons but not from adult rat brain (data not shown) [9]. This hints at the importance of CaBP5 during the development of the brain, however, its function has not been characterized yet. Although an NCS-1 mutant has been suggested to enhance the P/Q type Ca^{2+} currents in PC12 cells [26], the wild-type did not have any significant effect on the amplitude of I_{Ca} or exocytosis during first train of depolarizations [25]. Therefore, the inhibitory effects on the inward currents are related to the specific properties of L- and S-CaBP1.

Compared to S-CaBP1, L-CaBP1 has an extra sequence of 60 a.a. at the N-terminal. Here we show that both splicing isoforms have similar effects on inward currents and exocytosis, indicating that this segment is not the region responsible for these inhibitory activities. L-CaBP1 has been shown to modulate $Ca_v2.1$ and $Ca_v1.2$ by direct binding interaction [15, 27, 28]; it is possible that S-CaBP1 may have the same property needed to interact with these Ca^{2+} channels. The importance of this extra segment in L-CaBP1 needs to be further investigated.

The importance of L-, N-, and P/Q-type Ca^{2+} channels in exocytosis has been reported in bovine chromaffin cells [29]; the fraction of I_{Ca} carried by L-, N-, and P/Q-type channels has been suggested to be 20, 30, and 50%, respectively [30]. In our lab, 45.4 ± 2.6, 29.0 ± 2.3, and 25.6 ± 3.1% of the I_{Ca} was inhibited in the presence of ω-conotoxin GVIA (1 μM, N-type Ca^{2+} channel blcoker), ω-agatoxin GIVA (400 nM, P/Q-type Ca^{2+} channel blocker), and nifedipine (5 μM, L-type Ca^{2+} channel blocker), respectively (unpublished result). Our results demonstrate that the total I_{Ca} in cells expressing L- and S-CaBP1 was greatly inhibited, to 29 and 12% of control cells, respectively. These results hint that CaBP1s attenuate more than one type of Ca^{2+} currents. Furthermore, when coexpressed with L-CaBP1 in HEK293T cells, N-type Ca^{2+} currents are inhibited and the activation curve is positively shifted; these results directly demonstrate the inhibitory effects of CaBP1 on N-type Ca^{2+} channels. L-CaBP1 prevents Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels [15], but enhances the inactivation of P/Q-type Ca^{2+} channels [27]. Our results also hint that CaBP1 may enhance the inactivation of N-type Ca^{2+} channels. No matter how each subtype of Ca^{2+} channels is specifically modulated by the CaBP1s, the elevation in $[Ca^{2+}]_i$ elicited by membrane depolarization is inhibited. The effects of L- and S-CaBP1 on different types of Ca^{2+} channels will be further investigated.

The $[Ca^{2+}]_i$ responses evoked by membrane depolarization in neurons are attenuated by CaBP1s; how the Ca^{2+} channels are modulated is not clear. The large outward K^+ currents in neurons could not be completely blocked by the Cs^+ in the pipette solution and make the currents obtained turning outward above 0 mV (data not shown). Therefore, it is difficult to correctly estimate the Ca^{2+} currents in neurons esp. when the Ca^{2+} currents are inhibited; Ca^{2+} imaging could be an alternative way to monitor the Ca^{2+} signaling modulated by CaBP1s.

This report finds that both L- and S-CaBP1, when expressed in chromaffin and neuronal cells, inhibit the $[Ca^{2+}]_i$ elevation elicited by high K^+ stimulation. This can be explained by the inhibitory effects of CaBP1s on I_{Ca}; similarly, the decrease in exocytosis can be attributed to this factor as well [1]. Therefore, the physiological roles of CaBP1s in excitable cells are to attenuate stimulus-secretion coupling. Although the decrease in exocytosis can be mainly attributed to the inhibitory effect of CaBP1s on I_{Ca}, L-CaBP1 has also been demonstrated to associate with light chain 3, a component of the microtubules, in a Ca^{2+}-dependent manner [31]. In addition, L-CaBP1 has been shown to present on the secretory granules in pituitary cells [32]. The possible roles that L- and S-CaBP1 play in modulating the dynamics of the cytoskeleton so as to affect exocytosis, need to be further characterized.

Here we demonstrates the inhibitory effects of L- and S-CaBP1s on stimulus-secretion coupling in bovine chromaffin cells and the $[Ca^{2+}]_i$ responses in cortical neurons. Also, the inhibitory effects of L- and S-CaBP1 on I_{Na} hint at their modulatory effects on action potential firing, which is mainly determined by the opening of

Na^+ channels [33]. Furthermore, the colocalization of L-CaBP1 with L-type Ca^{2+} channels at the nerve terminal further supports the importance of CaBP1s in modulating synaptic activity [14]. Our findings reveal the importance of L- and S-CaBP1s in modulating stimulus-secretion coupling in excitable cells; the expression and activation of CaBP1s regulate the efficacy of synaptic transmission

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