The Calcium Sensor Protein Visinin-like Protein-1 Modulates the Surface Expression and Agonist Sensitivity of the α4β2 Nicotinic Acetylcholine Receptor*

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The calcium sensor protein visinin-like protein-1 (VILIP-1) was isolated from a brain cDNA yeast two-hybrid library using the large cytoplasmic domain of the α4 subunit as a bait. VILIP-1 is a myristoylated calcium sensor protein that contains three functional calcium binding EF-hand motifs. The α4 subunit residues 302–339 were found to be essential for the interaction with VILIP-1. VILIP-1 communopurified with detergent-solubilized recombinant α4β2 acetylcholine receptors (AChRs) expressed in tsA201 cells and with native α4 AChRs isolated from brain. Coexpression of VILIP-1 with recombinant α4β2 AChRs up-regulated their surface expression levels ~2-fold and increased their agonist sensitivity to acetylcholine ~3-fold. The modulation of the recombinant α4β2 AChRs by VILIP-1 was attenuated in VILIP-1 mutants that lacked the ability to be myristoylated or to bind calcium. Collectively, these results suggest that VILIP-1 represents a novel modulator of α4β2 AChRs that increases their surface expression levels and agonist sensitivity in response to changes in the intracellular levels of calcium.

Neuronal nicotinic acetylcholine receptors (AChRs) are members of a gene superfamily of ligand-gated ion channels. In vertebrates, neuronal AChRs are composed of subunits α2-α10 and β2-β4 (for review, see Ref. 1). AChR subunits have a large cytoplasmic domain between their third and fourth transmembrane domain whose amino acid sequence is highly divergent among the various subunits (2). The full functional unit of AChRs, like those of the N-methyl-D-aspartate receptors (3), is likely to include proteins that associate with this large cytoplasmic domain and modulate AChR functions.

To identify proteins associated with α4 AChRs, we used bait consisting of the large cytoplasmic domain of the α4 subunit to screen a mouse brain cDNA yeast two-hybrid library. In this paper, we describe the isolation of VILIP-1, a member of the visinin-like protein family of calcium sensor proteins, by such a screen. VILIP-1 is a member of a superfamily of neuronal calcium sensor proteins. This superfamily has been classified into the five subfamilies termed group I–V. The recoverins belong to group I, the frequenins and neuronal calcium sensor (NCS-1) to group II, the VILIPs, hippocalcins, and neurocalcins to group III, NCS-2 to group IV, and guanylyl cyclase-activating proteins and GC-inhibiting proteins to group V (for review, see Ref. 4).

The VILIP family is comprised of three members, VILIP-1, VILIP-2, and VILIP-3 (5–10). The members of this family contain 4 EF-hand motifs, of which only EF-hand 2, 3, and 4 are thought to be functional because EF-hand 1 lacks two oxygen-containing side chain residues crucial for binding calcium. A glycine residue at the second position on the polypeptide chain is myristoylated. Interestingly, within most, but not all members of this calcium sensor protein family, the myristoyl moiety is sequenced and exposed through a rapid conformational change that unmask in response to alterations in cellular levels of calcium, thus facilitating its association with membranes (11, 12). This calcium-dependent conformational switch has been termed the “calcium-myristoyl switch.”

In this paper we show that VILIP-1 interacts with both native and recombinant α4β2 AChRs. Coexpression of VILIP-1 with recombinant α4β2 AChRs increased their surface expression ~2-fold. This effect was significantly diminished for VILIP-1 mutants that are deficient in their ability to be myristoylated or bind calcium. Coexpression of VILIP-1 with recombinant α4β2 AChRs also increased their agonist-sensitivity ~3-fold. Collectively, these results support a novel role for VILIP-1 as an AChR-associated protein that modulates the surface expression levels and functional properties of α4β2 AChRs in response to changes in the intracellular levels of calcium.

EXPERIMENTAL PROCEDURES

Constructs—The rat α4 and β2 AChR subunit cDNA clones were generously provided by Dr. Stephen Heinemann (Salk Institute, San Diego, CA). All constructs were made by the PCR using appropriate pairs of forward and reverse synthetic oligonucleotide primers (Invitrogen) and Pfu Turbo (Stratagene, Inc., San Diego, CA). The restriction enzyme sites within all primers are underlined. The cDNA sequence

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§ The abbreviations used are: AChR, nicotinic acetylcholine (ACh) receptor; mAb, monoclonal antibody; EC50, half-maximal effective concentration of agonist; nεH, hill coefficient; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase A; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; NCS, neuronal calcium sensor; VILIP, visinin-like protein.
The generation of Recombinant AChRs in Human Embryonic Kidney tsA201 Cells—Human tsA201 cells, a derivative of human embryonic kidney cell line 293 were cultured in 6-well plates in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37 °C. Cells were transfected using LipofectAMINE 2000 (Invitrogen) at 90% confluence (~10^6 cells/well) with various combinations of cDNAs as per the manufacturer’s instructions. The absence of AChR subunit was detected after transfection if the cells were incubated at 37 °C for up to 72 h. Hence, cells were typically utilized after incubation at 30 °C for 24–48 h. This effect of lowering the temperature to increase recombinant α4β2 AChR subunit assembly efficiency has been previously reported (15, 16) and was found to be critical to get detectable levels of AChR surface expression. Hence, cells were treated ~16 h after transfection with or without forskolin (10 μM) or the PKA inhibitor H-89 (30 μM) and incubated at 30 °C for ~24 h and then for an additional 24 h at a physiological temperature of 37 °C before measuring surface AChRs.

**Immunolocalization of Recombinant AChRs in tsA201 Cells**—tsA201 cells were washed twice with ice-cold PBS containing 50 μM NaF and 1 mM sodium orthovanadate. Cells were then lysed in 1 ml of lysis buffer (50 mM NaCl, 30 mM tris(hydroxymethyl)aminomethane, pH 7.5, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidin, 2 mM sodium orthovanadate, 10 mM p-nitrophenylphosphate, 5 μg/ml aprotinin, 5 μg/ml leupetin, 5 μg/ml pepstatin, 0.3 μM okadaic acid, 1 mM sodium tetrathionate, 1 mM N-ethylmaleimide, 50 μM phenylarsonic oxide, and 1% Nonidet P-40) and agitated vigorously for 2 h at 4 °C. After centrifugation at 18,000 × g for 15 min, the clear supernatant from each sample (~1 ml) was incubated with 10 μl of mAb-coupled beads for 24 h. The beads were then washed 8 times with ~800 μl of solubilization buffer and eluted at 60 °C for 30 min with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 50 mM EDTA), and the eluted sample was incubated at 95 °C for 3 min. Analysis was performed by SDS-PAGE. The separated samples were then transferred onto a nitrocellulose membrane where the primary mAbs was detected using appropriate secondary Abs conjugated to horseradish peroxidase and visualized using horseradish peroxidase-conjugated goat anti-rat secondary Ab for 1 h followed by 3 washes with PBS and 10 min incubation with 50 μl of 5-bromo-4-chloro-3-indolyl phosphate/5-bromo-4-chloro-3-indolyl (BCIP/NBT) substrate. The membrane was then washed with PBS and exposed to X-ray film. After exposure, the membranes were soaked in 10% methanol/10% acetic acid for 15 min and developed with 50 μl of 30% hydrogen peroxide and 50 μl of 10% ammonium ferrous sulfite. The developed membranes were then exposed to X-ray film. The X-ray film was then scanned and the intensity of the bands was determined using a Gel Doc 2000 gel documentation system (Bio-Rad). The bands were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

**Expression in Xenopus Oocytes**—cDNAs were cloned into the vector pCS2+ (Invitrogen) but with a modified polylinker. cRNAs from linearized cDNA templates were synthesized in vitro using SP6 RNA polymerase in conjunction with reagents from the mMessage mMachine kit (Ambion, Austin, TX). Xenopus oocytes were prepared for injection as previously described (17). Oocytes were injected with cRNAs of the α4 and β2 subunits (20 ng/subunit) and of VII-1 (80 ng) per oocyte.
and incubated for 3–7 days at 16–18 °C in 50% L-15 medium (Invitrogen) containing 10 mM HEPES buffer, pH 7.5. 

Electrophysiological Recordings—Currents were measured using a standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725C) as previously described (17). Electrodes were filled with 3 M KCl and had resistances of 1.0–2.0 megohms for the voltage electrode and 0.5–0.1 megohm for the current electrode. All records were digitized at 200 Hz with MacLab software and hardware (AD Instruments). Data was analyzed using KALEIDOGRAF. The recording chamber was perfused at a flow rate of 10 ml/min with ND-96 solution (96 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.6).

Primary Cultures of Neurons—Primary cultures of rat cerebellar granule cells were isolated from postnatal day 6–8 male or female Sprague-Dawley rat pups. After rapid dissection, the cerebellum was immediately immersed in ice-cold calcium-magnesium-free PBS. The tissue was spun at 150 × g for 2 min, the supernatant was gently removed, and cells were dissociated by enzymatic treatment with Dnase in calcium-magnesium-free PBS and by repeated trituration through a series of decreasing diameter fire-polished Pasteur pipettes. Cells were resuspended in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 1× insulin-transferrin-selenium-S supplement (Invitrogen), 20 mM KCl, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells were plated at a density of 2–3 × 10⁶ cells/35-mm dish precoated with poly-l-lysine. Ara-C (10 μM) was added to the culture medium 16–18 h after plating to prevent the proliferation of non-neuronal cells. The cells were incubated at 37 °C in 5% CO₂ atmosphere for 6–7 days before being used in the experiments.

Immunohistochemistry—Cultured neurons were fixed with 100% methanol for 5 min, washed 3 times with 2 ml of PBS, and blocked using PBS containing 2% bovine serum albumin for 30 min. Cells were then incubated simultaneously with diluted anti-a subunit mAb (1/2000 dilution) and the anti-VILIP-1 antiserum (1/1000 dilution) in PBS containing 4% normal goat serum with gentle shaking overnight at 4 °C. Cells were washed 3 times for 15 min in PBS and then used for immunofluorescence microscopy.

Immunofluorescence Microscopy—Fluorescence microscopy was accomplished using a Leica DM RXA automated upright deconvolution microscope. Cells were scanned using 0.2-μm steps in the z axis, alternating between fluorescein isothiocyanate and rhodamine filters. The resulting optical section images were captured on Silicon Graphics workstations. Images were pseudocolored and processed using Adobe Photoshop software.

RESULTS

Isolation of VILIP-1 from a Brain Yeast Two-hybrid cDNA Library—we used the large cytoplasmic domain (residues 302–561) of the rat AChR α4 subunit as bait in the yeast two-hybrid system to screen ~10 × 10⁶ of a mouse brain λexA cDNA library. Unique clones thus identified were subject to limited nucleotide sequence (~100–150 nucleotides) analysis. By homology searches of established nucleotide databases, we identified VILIP-1 as a putative interacting protein.

Residues 302–339 of the α4 Subunit Are Essential for the Interaction of VILIP-1 with the α4 Cytoplasmic Domain—We used bait containing N-terminal and nested C-terminal deletions of the α4 cytoplasmic domain to test their ability to interact with VILIP-1 in the yeast two-hybrid system. We found using the nested C-terminal deletions that residues 302–339 of the α4 subunit loop are required for the interaction with VILIP-1. We verified this finding by using bait in which the N-terminal residues 302–339 were deleted and found that deletion of these residues was sufficient to abolish interaction of this bait with VILIP (Fig. 1).

VILIP-1 Interacts with Recombinant α4β2 AChRs in Transfected tsA201 Cells—To examine whether VILIP-1 could associate with recombinant α4β2 AChRs, we coexpressed VILIP-1 with recombinant α4β2 AChRs by transfecting tsA201 cells with their respective cDNAs. We immunoprecipitated 1% Nonidet P-40-solubilized recombinant α4β2 AChRs from tsA201 cells 48 h after transfection using anti-β2 subunit mAb-coupled beads. Proteins eluted from these beads were fractionated by SDS-PAGE, and the membrane containing the electroblotted proteins was probed for VILIP-1 using a polyclonal antiserum to VILIP-1. As controls for nonspecific binding of proteins to beads, we used rat IgG-coupled beads. We found that VILIP-1 coimmunoprecipitated with recombinant α4β2 AChRs, and no immunoreactivity for VILIP-1 was observed with the control rat IgG beads (Fig. 2). The lysates represent ~1/250 of the total solubilized protein used in each of the coimmunopurifications.

The relatively weaker signal from VILIP-1 compared with the α4 and β2 signals in the coimmunoprecipitation experiments suggests that only a small fraction of the total VILIP-1 present is associated with the α4β2 AChR complexes under these conditions. It is likely that the detergent used to solubilize the α4β2 AChR complexes significantly affects protein-protein interactions, including that of VILIP-1, with α4β2 AChRs.

To further study if myristoylation and the calcium binding EF-hand motifs were required for the association of VILIP-1 with the α4β2 AChRs, we generated two VILIP-1 mutants. The first mutant, mVILIP-myr, was not myristoylated because the residue at which it is myristoylated (Gly-2) was mutated to Ala.

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VILIP-1 has four EF-hand motifs, and their structure consists of two α-helical segments bridged by a calcium binding loop. The loop is formed by 12-amino acid stretches, 6 of which (designated as X, Y, Z, −Y, −X, −Z at positions 1, 3, 5, 7, 9, and 12) participate in the coordination of one calcium ion. The N-terminal-most EF-hand motif I has substitutions at the conserved positions that are essential for binding calcium ions, and hence, only three of the four EF-hand motifs are thought to be capable of binding calcium ions. Thus, the second mutant generated was mutated at position −X within each of the three functional calcium binding EF-hands (D81V/T117A/T167A) to yield the triple mutant mVILIP-2,3,4EF. Both these mutants, mVILIP-myr and mVILIP-2,3,4EF, also interacted with recombinant α4β2 AChRs albeit with some apparent loss in association (as inferred from the intensity of band on immunoblots in Fig. 2). This result showed that calcium is not essential for the binding of VILIP-1 to α4β2 AChRs. This result is similar to that found for other members of this superfamily of calcium sensor proteins (KChIPs), which also exhibit a lack of calcium dependence for their association with A-type K⁺ channels (18).

The expression of VILIP-1 with α4β2 AChRs increases their surface expression. We examined the cell surface expression of α4β2 AChRs coexpressed with VILIP-1 by measuring their surface expression levels using a previously described enzyme-linked immunosassay (15, 16). As controls for nonspecific binding of mAbs, we used cells transfected with the vector alone. The surface expression of wild-type α4β2 AChRs coexpressed with VILIP-1 was found to be increased ~2-fold over those cells expressing α4β2 AChRs alone. We also examined the ability of both mutants mVILIP-myr and mVILIP-2,3,4EF to increase surface expression of α4β2 AChRs and found that both mVILIP-myr and mVILIP-2,3,4EF did not significantly change the expression levels of α4β2 AChRs when coexpressed with them in tsA201 cells (Fig. 3).

The increase in surface expression of α4β2 AChRs by VILIP-1 is not due to changes in cAMP levels or activation of PKA—VILIP-1 has previously been shown to cause small changes in the intracellular levels of cAMP when expressed as a recombinant protein in cells (19). We attempted to determine if this relatively small increase in cAMP levels or the subsequent activation of PKA could be responsible for the up-regulation of α4β2 AChRs by VILIP-1. We measured the surface expression of α4β2 AChRs in the presence and absence of VILIP-1 in cells pretreated with and without forskolin (10 μM) or with and without the PKA inhibitor H89 (30 μM). We observed that treatment of cells with forskolin alone up-regulated α4β2 AChR surface expression as previously described (20). In cells coexpressing VILIP-1, VILIP-1 also increased α4β2 AChR surface expression in addition to that observed by forskolin alone (Fig. 4, center panel). The PKA inhibitor H89 failed to block the ability of VILIP-1 to up-regulate α4β2 AChRs (Fig. 4, right panel). These results support our conclusion that the up-regulation of α4β2 AChR by VILIP-1 occurs through a mechanism that is distinct from that attributed to changes in cellular levels of cAMP or the activation of PKA-dependent phosphorylation.

VILIP-1 increases the agonist sensitivity of α4β2 AChRs expressed in oocytes—We coexpressed α4β2 AChRs with or without VILIP-1 from in vitro transcribed cRNAs in oocytes to determine whether VILIP-1 altered the functional properties of AChRs. Currents were elicited by 4-s application of ACh and measured using the 2-electrode voltage clamp technique. Both α4β2 AChRs and α4β2 AChRs coexpressed with VILIP-1 gave concentration/response curves that were best fit by a one-site Hill equation (Fig. 5, top panel). ACh activated α4β2 AChRs with an EC₅₀ = 32 ± 7 μM (n₄₄ = 0.95) and α4β2 AChRs coexpressed with VILIP-1 with an EC₅₀ = 13 ± 3 μM (n₄₄ = 0.95). Thus, the ACh sensitivity was found to be ~3-fold higher for α4β2 AChRs coexpressed with VILIP-1 compared with α4β2 AChRs expressed alone. ACh activated α4β2 AChRs coexpressed with mVILIP-myr with an EC₅₀ = 26 ± 8 μM (n₄₄ = 0.6), and α4β2 AChRs coexpressed with mVILIP-2,3,4EF with an EC₅₀ = 7 ± 2 μM (n₄₄ = 0.46) (Fig. 5, bottom panel). The significantly lower n₄₄ observed for the single-site fits for the α4β2 AChR mutants were also observed with two-site fits and are consistent with fits to responses from more than one population of functional α4β2 AChR. The increased agonist sensitivity of α4β2 AChRs coexpressed with VILIP-1 is not due to the ability of VILIP-1 to slightly increase cAMP levels in cells, because we have previously observed no significant change in the agonist sensitivity of α4β2 AChRs expressed in oocytes pretreated with forskolin (50 μM, 4 h) (15). Thus, our results support our conclusion that the change in agonist sensitivity of α4β2 AChRs is due to the direct intracellular modulation of α4β2 AChRs by VILIP-1.

VILIP-1 coimmunoprecipitates with native detergent-solubilized α4β2 AChRs from brain—to investigate the physiolog-
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VILIP-1 up-regulates α4β2 AChRs through a non-cAMP-dependent mechanism. The surface expression levels of α4β2 AChRs was determined in cells coexpressing VILIP-1, mVILIP-myr, and mVILIP-2,3,4EF. The cells were untreated (Control) or pretreated with forskolin (10 μM) and the PKA inhibitor H-89 (30 μM), and the AChR surface expression levels were measured after 48 h. The measurements in each experiment were done in duplicate, and the error bars represent the S.E. of measurement from at least two separate experiments.

Fig. 4. VILIP-1 up-regulates α4β2 AChRs through a non-cAMP-dependent mechanism. The surface expression levels of α4β2 AChRs in transfected oocytes were clamped at a holding potential of −70 mV. Currents elicited by a 4-s application of different concentrations of ACh were recorded with a 4-min wash-out period between each application. Data obtained from 3–4 oocytes from at least two independent experiments were normalized to the control response elicited by 1 mM ACh, averaged, and fit using the Hill equation. The error bars represent the S.E. of measurement from at least two separate experiments.

Fig. 5. VILIP-1 increases the agonist-sensitivity of α4β2 AChRs expressed in oocytes. α4β2 AChRs were expressed with and without wild-type VILIP-1 or the mutants VILIP-myr and mVILIP-2,3,4EF from in vitro transcribed cRNAs in Xenopus oocytes. The oocytes were clamped at a holding potential of −70 mV. Currents elicited by a 4-s application of different concentrations of ACh were recorded with a 4-min wash-out period between each application. Data obtained from 3–4 oocytes from at least two independent experiments were normalized to the control response elicited by 1 mM ACh, averaged, and fit using the Hill equation. The error bars represent the S.E. of measurement from at least two separate experiments.

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protein VILIP-1. VILIP-1 coimmunoprecipitates with both recombinant and native α4β2 AChRs and shows partial colocalization with native α4 subunits in cultured cerebellar granule cells. Coexpression of VILIP-1 with α4β2 AChRs in tsA201 cells increases the surface expression of α4β2 AChRs ~2-fold. Coexpression of VILIP-1 with recombinant α4β2 AChRs increases their agonist sensitivity ~3-fold. These results suggest a novel mechanism by which changes in intracellular levels of calcium can alter the expression levels and functional properties of AChRs.

The surface expression of α4 AChRs can be modulated by different mechanisms. The binding of nicotine to α4 AChRs up-regulates their surface expression levels both in vitro (20, 29–31) and in vivo (32–35). Our results illustrate a novel mechanism by which α4β2 AChR surface expression can be up-regulated by an endogenous cytosolic protein. The exact mechanism by which the association of VILIP-1 with α4β2 AChRs increases their surface expression remains to be established. However, we have provided experimental evidence that the previously reported ability of recombinant VILIP-1 to increase basal levels of cAMP or the coupled PKA activity is not responsible for the increase in α4β2 AChR surface expression. This is because we observe that VILIP-1 increases the surface expression of α4β2 AChR even in cells that have been pretreated with forskolin and in cells that have been pretreated with the PKA inhibitor H-89. Because we observe little detectable colocalization of VILIP-1 with α4 subunits in the endoplasmic reticulum/Golgi membranes, it is unlikely that VILIP-1 is involved in the trafficking of α4 AChRs to the surface membrane. The colocalization at the surface membrane instead favors the possibility that VILIP-1 alters the turnover of α4 AChRs at the surface membrane when it associates with them after the activation of its calcium-myristoyl switch and translocation to the membrane in response to changes in intracellular levels of calcium. The ability of VILIP-1 to increase the surface expression of membrane proteins it associates with is conserved among other members of this superfamily because KChIP also increases the surface density of A-type K+ channels (18).

The VILIP-1 mutants lacking the myristoyl moiety and the functional EF-hand motifs were attenuated in their ability to modulate the α4β2 AChR response. This was inferred from the Hill fits that gave significantly lower nH for both single-site and two-site fits, suggesting that the heterogeneous responses were from modulated and unmodulated α4β2 AChRs, possibly because of the loss in each of the VILIP-1 mutant’s affinity for the α4β2 AChR. We observed that ACh activated α4β2 AChR coexpressed with mVILIP-2,3,4EF with an EC50 that was significantly lower than the EC50 for activating α4β2 AChR cox-
pressed with mVILIP-myr. We have recently observed that mVILIP-myr is poorly associated with membranes even in the presence of calcium in contrast to the association of mVILIP-2,3,4EF with membranes both in the presence and absence of calcium (13), possibly because the myristoyl moiety is constitutive exposed due to structural changes caused by mutations in the EF-hand motifs. Based on these results we concluded that the calcium binding EF-hand motifs of VILIP-1 have a structural role in triggering the calcium-myristoyl switch of VILIP-1, but its ability to remain associated with membranes critically requires the presence of the myristoyl moiety. Thus, the differences between the EC50 values for activation of the αβ2 AChR coexpressed with the two VILIP-1 mutants most likely reflected individual differences in their ability to associate with membranes (loss for mVILIP-myr and retention for mVILIP-2,3,4EF).

Neuronal AChRs in the central nervous system are the primary mediators of addiction to nicotine. Addiction to nicotine due to repetitive activation of AChRs is thought to change the functional properties of the AChRs themselves and the functional circuitry of the neuronal system by a complex sequence of molecular and cellular processes similar to those activated by other drugs of addiction (36–38). Because AChRs exhibit significant permeability to calcium, it is reasonable to expect that some of these changes are effected via calcium entry through nicotine-gated AChRs. It was recently demonstrated that chronic low doses of nicotine can also up-regulate recombinant αβ2 AChR function by increasing their agonist sensitivity (39). Interestingly, this result was attributed to a possible interaction of αβ2 AChRs with cytosolic proteins in the host cells (39). However, little is known about the endogenous intracellular proteins that can increase the agonist sensitivity of αβ2 AChRs. The ability of VILIP-1 to increase the agonist sensitivity of αβ2 AChRs could account for the observations of that chronic nicotine alters αβ2 AChR function. The increased agonist sensitivity of αβ2 AChRs may represent one mechanism by which chronic exposure to low doses of nicotine (as occurs in the central nervous system neurons of nicotine addicts) augments synaptic transmission involving αβ2 AChRs where they, in turn, modulate the release of multiple neurotransmitters including dopamine.

The illustration in Fig. 8 shows a model that incorporates key features of our findings. In this model, repetitive exposure to nicotine would increase calcium entry into neurons due to activation of nicotine-gated AChRs, which in turn would trigger the calcium-myristoyl switch of VILIP-1 and cause it to translocate to the membrane in the vicinity of activated AChRs. The association of VILIP-1 with the AChRs would have two consequences, 1) to increase AChR levels possibly by decreasing their turnover and 2) to stabilize them in a state with higher agonist sensitivity. It is possible that behaviors that lead to accumulation of AChRs in this state, including smoking tobacco, become reinforced because of subsequent functional adaptations of neural networks to functioning with AChRs that have higher agonist sensitivity.

We have previously demonstrated that a chaperone protein identified as 14-3-3 has a dynamic role in regulating the expression levels of αβ2 AChRs through a phosphorylation-dependent interaction with the α subunit (15). In addition, an endogenous prototoxin identified as lynx1 was shown to decrease the agonist sensitivity of αβ2 AChRs and slow their recovery from desensitization (40). The ability of VILIP-1 to modulate both the surface expression and the agonist sensitiv-