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Spatial association of the Cav1.2 calcium channel with $\alpha_5\beta_1$ -integrin

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Chao J, Gui P, Zamponi GW, Davis GE, Davis MJ. Spatial association of the Cav1.2 calcium channel with $\alpha_5\beta_1$ -integrin. *Am J Physiol Cell Physiol* 300: C477–C489, 2011. First published December 22, 2010; doi:10.1152/ajpcell.00171.2010.—Engagement of $\alpha_5\beta_1$ -integrin by fibronectin (FN) acutely enhances Cav1.2 channel (Ca_L) current in rat arteriolar smooth muscle and human embryonic kidney cells (HEK293-T) expressing Ca_L . Using coimmunoprecipitation strategies, we show that coassociation of Ca_L with α_5 - or β_1 -integrin in HEK293-T cells is specific and depends on cell adhesion to FN. In rat arteriolar smooth muscle, coassociations between Ca_L and $\alpha_5\beta_1$ -integrin and between Ca_L and phosphorylated c-Src are also revealed and enhanced by FN treatment. Using site-directed mutagenesis of Ca_L heterologously expressed in HEK293-T cells, we identified two regions of Ca_L required for these interactions: 1) COOH-terminal residues Ser¹⁹⁰¹ and Tyr²¹²², known to be phosphorylated by protein kinase A (PKA) and c-Src, respectively; and 2) two proline-rich domains (PRDs) near the middle of the COOH terminus. Immunofluorescence confocal imaging revealed a moderate degree of wild-type Ca_L colocalization with β_1 -integrin on the plasma membrane. Collectively, our results strongly suggest that 1) upon ligation by FN, Ca_L associates with $\alpha_5\beta_1$ -integrin in a macromolecular complex including PKA, c-Src, and potentially other protein kinases; 2) phosphorylation of Ca_L at Y²¹²² and/or S¹⁹⁰¹ is required for association of Ca_L with $\alpha_5\beta_1$ -integrin; and 3) c-Src, via binding to PRDs that reside in the II–III linker region and/or the COOH terminus of Ca_L , mediates current potentiation following $\alpha_5\beta_1$ -integrin engagement. These findings provide new evidence for how interactions between $\alpha_5\beta_1$ -integrin and FN can modulate Ca_L entry and consequently alter the physiological function of multiple types of excitable cells.

focal adhesion complex; fibronectin; c-Src; proline-rich domain; protein kinase A

INTEGRINS ARE HETERODIMERIC transmembrane proteins that facilitate adhesion and communication of cells with their surroundings through binding to extracellular matrix proteins (ECM). Extensive studies implicate an essential role for integrins in the transmission of mechanical forces at focal adhesion sites (23, 25, 30). Integrin-mediated mechanotransduction is accomplished by activation of signaling molecules, such as focal adhesion kinase (FAK) and other tyrosine kinases, including c-Src, in conjunction with the recruitment of multiple proteins to focal adhesions and reorganization of the cytoskeleton (36). Integrins thereby translate physical forces into biochemical signals that ultimately alter cellular function.

Ca^{2+} entry through the L-type voltage-gated Cav1.2 calcium channel (Ca_L) is required for stretch-induced contraction of vascular smooth muscle (VSM). Intracellular pressurization of isolated arterial myocytes leads to enhancement of Ca_L current,

suggesting that Ca_L may be modulated by mechanical stress applied to the plasma membrane (22). A link between Ca_L and integrins in transducing mechanical force has also been demonstrated in arterioles. The application of peptides containing an Arg-Gly-Asp (RGD) sequence, a cryptic binding motif in ECM that becomes exposed during vascular injury (7), inhibits pressure-dependent myogenic tone, through interactions with multiple VSM and endothelial cell integrins (8). Moreover, function-blocking antibodies against α_5 -, β_1 -, and β_3 -integrin significantly reduce the degree of myogenic constriction to pressure elevation (29). The effects of integrin antibodies on myogenic tone are mediated in part by altered Ca^{2+} entry through Ca_L because electrophysiological studies reveal selective effects of integrin ligands on Ca_L current; the engagement and clustering of $\alpha_5\beta_1$ -integrin by insoluble fibronectin (FN) or anti- α_5 -integrin antibody acutely potentiates Ca_L current, whereas engagement of $\alpha_v\beta_3$ -integrin by vitronectin or soluble ligands inhibits Ca_L current in isolated VSM cells (41). Using site-directed mutagenesis strategies, two phosphorylation sites within the COOH terminus of the α_{1C} -subunit of Ca_L (α_{1C} - Ca_L) were identified as targets of protein kinase A (PKA) and c-Src that become activated downstream from $\alpha_5\beta_1$ -integrin ligation (14).

A critical remaining issue is the extent to which $\alpha_5\beta_1$ -integrin, PKA, c-Src, and Ca_L are spatially coupled within a cell. In the present study, we tested the hypothesis that $\alpha_5\beta_1$ -integrins spatially associate with Ca_L and that this association is required to modulate Ca_L function. Using human embryonic kidney cells (HEK293-T) overexpressing the neuronal isoform of Ca_L and VSM cells from rat skeletal muscle arterioles as model systems, the interactions between endogenous $\alpha_5\beta_1$ -integrin and Ca_L and between c-Src and Ca_L were examined using immunoprecipitation (IP), immunoblotting (IB), immunofluorescence confocal microscopy, and patch-clamp methods.

METHODS

Cell culture and transient transfection. HEK293-T/(TSA201) cells were maintained in 10% FBS (Hyclone)-DMEM (high glucose) supplemented with 2 mM glutamine, 100 units of penicillin, and 100 μg of streptomycin in 5% CO_2 at 37°C. When cells reached 70–80% confluence, wild-type (WT) or mutant neuronal Cav1.2 calcium channel (Ca_L) cDNAs composed of α_{1C-c} (6 μg), β_{1b} (3 μg), and $\alpha_2\delta$ (2 μg) subunits, in a total volume of 14 μl , were transfected into HEK293-T cells grown in 60-mm tissue culture dishes containing 2.5 ml of DMEM with Lipofectamine 2000 (20 μl) for 18–24 h. Green fluorescent protein (GFP; 0.5–1 μg) was cotransfected with Ca_L to monitor transfection efficiency. Untransfected cells were used as a control. Cells were incubated for another 24 h posttransfection before passing and plating onto either FN (catalog no. 354403, BD BioCoat, BD Biosciences), BSA (2%), or poly-L-lysine-treated plates for 1 h, followed by protein isolation or immunofluorescence imaging. To account for variation in the expression level of α_{1C} - Ca_L due to

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variation in transfection efficiency among the different Ca_L mutants, the experiments for all Ca_L mutants were performed in parallel with WT- Ca_L transfection using the same passage of cells.

For electrophysiological experiments, HEK293-T cells were transfected using calcium phosphate, after which patch-clamp recordings were performed 48–72 h posttransfection as previously described (14). Only single cells expressing GFP were used for electrophysiological protocols.

The various mutations in the α_{1C} -COOH terminus were generated using site-directed mutagenesis as described previously (14) and as illustrated in Fig. 1.

Isolation of skeletal muscle arterioles. The isolation of first- and second-order arterioles from rat cremaster muscle was performed as described previously (21, 40, 41), except for addition of $1\times$ Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) into the dissection chamber immediately before dissection. All animal protocols were approved by the University of Missouri Animal Care and Use Committee and conformed to the Public Health Service Policy for the Humane Care and Use of Laboratory Animals (PHS Policy, 1996).

Electrophysiological recording. Patch-clamp recordings were made using an EPC9 amplifier under the control of Pulse software (HEKA Instruments) in the conventional whole cell mode, as previously described (14). Pipettes were filled with Cs^+ pipette solution containing (in mM) 110 CsCl, 20 TEA chloride, 10 EGTA, 2 MgCl_2 , 10 HEPES, and 1 CaCl_2 (pH 7.2 with CsOH). Ba^{2+} was used as the charge carrier to increase the magnitude of the inward current and to minimize calcium-dependent current inactivation. Cs^+ was used to block endogenous K^+ current.

Immunoprecipitation and immunoblotting. Proteins from HEK293-T cells were isolated as described by Ling et al. (27) with minor modifications. The lysis buffer was composed of 1% Triton X-100 containing the following: 2.5 mM Tris, pH 7.4, 13.8 mM NaCl, 0.3 mM KCl, 2 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM benzamide, 1 mM Na_3VO_4 , 4 mM Pefabloc, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ of calpain I and II, and 10 $\mu\text{g}/\text{ml}$ pepstatin A. Isolation of total proteins in arterioles was performed as described above except for using 0.25% Triton X-100, along with 2% *n*-octyl- β -D-glucopyranoside to lyse arterioles. Arterioles (1st order and 2nd order) were snap-frozen in liquid nitrogen after dissection. Vessels were cut into smaller pieces and pulverized with a plastic pestle in 0.65-ml centrifuge tubes in the presence of an optimal amount of lysis buffer. Tubes containing arterioles were frequently dipped into liquid nitrogen and kept on dry ice during pulverization. Three hundred to four hundred micrograms of total protein (150–250 μg for arterioles) were precleared with protein A/G agarose beads (catalog no. SC-2003; Santa Cruz) in the presence of either rabbit or mouse IgG

(catalog no. PP64 and PP54, respectively; Millipore), followed by overnight IP in lysis buffer at 4°C with one of the following antibodies: 1) either rabbit anti-Cav1.2 polyclonal antibody (Ab) (3.2 μg ; catalog no. AB5156, Millipore) or pan Calcium Channel Ab (catalog no. Ab6298, Abcam), recognizing the α_{1C} -subunit of Ca_L (α_{1C} - Ca_L); 2) mouse anti-human β_1 -integrin monoclonal Ab (5 μg , clone B3B11, catalog no. MAB2251Z, Millipore); 3) purified mouse anti-human α_5 -integrin Ab (5 μg , clone 1/CD49e, catalog no. 610634, BD Biosciences); 4) mouse anti-pp60c-Src (4 μg , clone GD11, catalog no. 05-184, Millipore); or 5) anti-phosphorylated-Tyr Ab (5 μg , clone 4G10, catalog no. 05-321, Millipore). The immune complex was captured using either protein A (catalog no. SC-2001, Santa Cruz) or protein G agarose beads (catalog no. 17088601, Amersham) prior to SDS-PAGE (6%), followed by overnight transfer to nitrocellulose membrane (0.45 nm) and IB. To confirm binding specificity, the whole cell lysate was also immunoprecipitated with 1) control IgG for the appropriate host species and captured by protein A or protein G beads, or 2) protein A or protein G beads alone. Equal loading of total protein was confirmed by Ponceau staining (Sigma) before IB. Ca_L was detected using rabbit anti- Ca_L α_{1C} -subunit Ab (catalog no. AB5156, 1:6,500 dilution) for HEK293-T cells or pan Calcium Channel Ab (1:2,500 dilution) for rat arterioles. Endogenous β_1 -integrin was detected using mouse anti-human β_1 -integrin Ab or rabbit anti-rat β_1 -integrin Ab (catalog no. MAB2251Z, 1:1,400 dilution and catalog no. AB1952P, 1:1,500 dilution, respectively; Millipore). c-Src or phosphorylated c-Src was detected using anti-pp60c-Src (catalog no. 05-184, 1:1,200 dilution, Millipore) or rabbit anti-rat phospho-Src family (Tyr416) Ab (catalog no. 2101S, 1:1,000 dilution, Cell Signaling Technology), respectively. The immune complexes were incubated with either goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated antibody (H&L, 1:120,000 dilution, catalog no. AP132P, Millipore) or donkey anti-mouse-IgG-HRP-conjugated antibodies (H&L, 1:60,000 dilution, catalog no. 715-035-150, Jackson Research Laboratory) followed by detection with Pierce SuperSignal extended Dura or Femto substrate (Thermo Scientific). After probing, the blot was stripped with Restore Western blot stripping buffer (Thermo Scientific) and reprobed with either anti- α_{1C} - Ca_L , anti- β_1 -integrin, or anti-c-Src Ab to assess the relative loading. For detection of the immune complex from arterioles, the blot was processed as described above except that SuperSignal Western Blot Enhancer (catalog no. 46641, Thermo Scientific) was used according to the manufacturer's instruction before IB. The results were captured by exposure to Kodak Biomax MR, MS, or Light film (Sigma), and the band intensity for the protein of interest was quantified using Bio-Rad Quantity One. Quantification of co-IP was determined as described by Cherubini et al. (4). The ratio of immunoprecipitated α_{1C} - Ca_L to β_1 -integrin in WT- α_{1C} - Ca_L -expressing cells was set as 1. The ratio of immunoprecipitated α_{1C} - Ca_L to β_1 -integrin in mutant α_{1C} - Ca_L -expressing cells was expressed relative to WT- α_{1C} - Ca_L .

Immunofluorescence confocal imaging. To determine the distribution and expression of α_{1C} - Ca_L and β_1 -integrin, cells were plated on poly-L-lysine or FN-coated tissue culture dishes with coverglass bottoms (Fluorodish, catalog no. FD35-100, WPI) for 1 h after 24 h of transfection, followed by fixation with 2% paraformaldehyde and staining as described previously (40). α_{1C} - Ca_L was determined using the same antibody for IP, followed by donkey anti-rabbit IgG conjugated to Alexa 488 (1:1,600 dilution, catalog no. A21206, Invitrogen). β_1 -Integrin was detected using mouse anti-human β_1 -integrin Ab (1:75 dilution, clone HUTS 21, catalog no. 556037, BD Biosciences), followed by goat anti-mouse IgG conjugated with Alexa 555 (1:1,400 dilution, catalog no. A21424, Invitrogen). Counterstaining of nuclei was performed using TO-PRO-3 (0.5 μM , catalog no. T3605, Invitrogen). Staining of paxillin or vinculin was determined using rabbit anti-paxillin polyclonal Ab (1:300 dilution, catalog no. AB3794, Millipore) or mouse anti-vinculin antibody (clone V284, 1:80 dilution; catalog no. 05-386, Millipore). Cells were incubated with donkey anti-rabbit IgG conjugated with Alexa 488 alone or goat anti-mouse

Fig. 1. Schematic depicting the domain structure of wild-type (WT) L-type voltage-gated Cav1.2 calcium channel (Ca_L) α_{1C} -subunit and mutant α_{1C} - Ca_L constructs used in this study. I–IV, the four transmembrane repeat of α_{1C} . EF and DCT, EF hand motif and the distal COOH terminus inhibitory domain; P, proline-rich domain; truncated α_{1C} - Ca_L , WT α_{1C} - Ca_L missing the distal COOH-terminal 280 amino acids; $\Delta\text{P1}/\Delta\text{P2}$ α_{1C} - Ca_L , WT α_{1C} - Ca_L missing the two proline-rich domains (aa 1955–1959; aa 1967–1973); $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ α_{1C} - Ca_L , two single-point mutations at serine (S^{1901}A) and tyrosine (Y^{2122}F) in full-length neuronal WT- Ca_L .

IgG conjugated to Alexa 555 alone, or the combination of both as a negative control to confirm the specificity of staining. ProLong Gold Antifade Reagent (catalog no. P36934, Invitrogen) was applied to the sample after staining, followed by storage at -30°C until images were captured.

Immunofluorescence images were taken using a laser-scanning confocal system (Leica TCS SP5 Microsystems) attached to an upright microscope (Leica DM 6000 CS) and were collected using a $\times 63$ oil objective (numerical aperture 1.42). Excitation of the fluorophores was achieved using argon 488, He/Ne 543, or He/Ne 633 nm laser combinations with pinhole size set at 1 airy disk. Images were taken at 512×512 pixels with a step size of $0.2 \mu\text{m}$ and a zoom factor of 5. The image size was $\sim 49.2 \mu\text{m}^2$.

To prevent bleed-through of different wavelengths that might interfere with colocalization analysis, all the images were taken using a sequential acquisition procedure at wavelengths of 488/519 nm (excitation/emission) for the α_{1C} -Ca_L-subunit; 543/569 nm (excitation/emission) for β_1 -integrin, and 633/656 nm (excitation/emission) for nuclear staining.

Quantification of α_{1C} -Ca_L association with β_1 -integrin. The association of α_{1C} -Ca_L with β_1 -integrin was analyzed with ImageJ software (version 1.39d, National Institutes of Health, Bethesda, MD). The degree of α_{1C} -Ca_L association with β_1 -integrin was quantified using Pearson's coefficient, Mander's coefficient, and intensity correlation analysis (ICA) (24, 33). The advantage of using ICA analysis is that it avoids taking the overlap of randomly distributed proteins into account for colocalization if the intensity of the two target proteins does not vary coincidentally (24). ICA values are distributed between -0.5 and $+0.5$. ICA values close to 0 indicate random staining, values $-0.5 \leq \text{ICA} < 0$ represent segregated staining, and values $0 < \text{ICA} \leq +0.5$ represent interdependent staining. The degree of α_{1C} -Ca_L association with β_1 -integrin was normalized to the average ICA value for the coassociation of paxillin with vinculin (0.25), which was shown previously to be very strong (37).

Statistical analysis. Results are presented as means $\pm$ SE from at least three independent experiments for IP or four independent experiments for immunofluorescence confocal imaging (IF). Statistical differences were analyzed using GraphPad InStat or Prism (version 3.06 and version 5.0, respectively; GraphPad, San Diego, CA) with either analysis of variance or unpaired *t*-tests. $P < 0.05$ was considered significant.

RESULTS

Ca_L association with $\alpha_5\beta_1$ -integrin depends on adhesion to fibronectin. To determine whether α_{1C} -Ca_L associated with $\alpha_5\beta_1$ -integrin, IP of whole cell lysates from HEK293-T cells expressing the neuronal Ca_L using either anti- β_1 - or anti- α_5 -integrin antibody was followed by IB to probe for the presence of α_{1C} -Ca_L. The neuronal Ca_L isoform was used as a model system because α_{1C} -Ca_L mutants had been previously generated by site-directed mutagenesis strategies; neuronal and smooth muscle (SM) cell (SMC) Ca_L isoform share 90% homology in amino acid sequence, and we previously demonstrated that heterologously expressed rat neuronal and SMC isoforms (Cav1.2c and Cav1.2b, respectively) showed a similar degree of current potentiation following $\alpha_5\beta_1$ -integrin ligation (14). We first determined the association between α_{1C} -Ca_L and β_1 -integrin in cells plated on poly-L-lysine-treated dishes. As shown in Fig. 2A, no appreciable α_{1C} -Ca_L was observed after IP using anti- β_1 -integrin Ab in either control (untransfected cells, lane 2) or cells expressing WT α_{1C} -Ca_L (lane 7) when plated on poly-L-lysine-treated dishes. However, when cells expressing WT α_{1C} -Ca_L were plated on FN (Fig. 2B), α_{1C} -Ca_L was detected after IP of the lysate using either anti- α_5 - (lane 4) or anti- β_1 -integrin antibody (lane 3). No specific α_{1C} -Ca_L band was observed in cells expressing α_{1C} -Ca_L plated on BSA-coated dishes after IP using anti- β_1 -integrin or anti-c-Src Ab (Supplemental Fig. S1A, lanes 6 and 7, respectively) or after IP of the lysate using mouse IgG or protein-G (Fig. 2B, lanes 2, 5, and 9). These results indicate that the association of α_{1C} -Ca_L with α_5 or β_1 -integrin in HEK293-T cells is specific and depends on cell adhesion to FN.

To confirm that the effect of FN on α_{1C} -Ca_L association with β_1 -integrin was not an artifact of Ca_L overexpression in HEK293-T cells, we examined the same interaction in lysates from arteriolar SM. Arterioles were dissected and then incubated with exogenous FN abuminally (300 $\mu\text{g}/\text{ml}$) for 1 h. When arteriolar SM lysates were immunoprecipitated with

A HEK293-T cells plated on poly-lysine

B HEK293-T cells plated on fibronectin

Fig. 2. Ca_L association with α_5 - or β_1 -integrin depends on adhesion of human embryonic kidney cells (HEK293-T) to fibronectin (FN). **A**: representative blot showing coimmunoprecipitation (co-IP) protocol on protein lysed from control (untransfected) or neuronal WT-Ca_L-expressing HEK293-T cells plated onto poly-L-lysine-treated dishes. No α_{1C} -Ca_L appears to be pulled down by IP with anti- β_1 -integrin antibody (Ab) in either control (untransfected) or WT Ca_L-expressing cells (lanes 2 and 7, respectively). Expression of α_{1C} -Ca_L was confirmed in cells expressing WT-Ca_L by immunoblotting (IB) with anti- α_{1C} -Ca_L Ab, showing a specific band at molecular mass 190 to 240 kDa (lane 4). **B**: representative blot showing pull-down of α_{1C} by anti- α_5 or anti- β_1 -integrin Ab (lanes 4 and 3, respectively) only from cells expressing WT-Ca_L, not control (untransfected) cells (lanes 7 and 8, respectively), when plated onto FN-coated plates before IP and IB. All blots were repeated at least three times. Prot-G, protein-G.

anti- α_{1C} -Ca_L, β_1 -integrin was detected only in arterioles incubated with FN (Fig. 3A, lanes 1 and 2), compared with control (lanes 5 and 6). The same blot was also stripped and reprobed for the expression of α_{1C} -Ca_L. In addition, incubation with FN did not substantially change the level of α_{1C} -Ca_L expression in arterioles (Supplemental Fig. S1B). These results indicate that the association of α_{1C} -Ca_L with β_1 -integrin in SMC depends on ligation of β_1 -integrin by FN.

c-Src, a tyrosine kinase activated upon integrin ligation by ECM ligands (18), has been shown to modulate Ca_L function in SMC (2, 3, 19, 39). Thus, we also examined whether c-Src associated with α_{1C} -Ca_L in arteriolar SM and whether FN was important for this association. As shown in Fig. 3B, c-Src was detected in immunoprecipitates using two different anti- α_{1C} -Ca_L antibodies (Fig. 3B, lanes 1 and 2). However, abluminal incubation with FN did not substantially change the degree of c-Src association with α_{1C} -Ca_L (Fig. 3B, lanes 1 and 2 vs. lanes 5 and 6). Next, we tested whether incubation with FN altered the phosphorylation status of c-Src in association with α_{1C} -Ca_L. A basal level of phosphorylated c-Src was observed in untreated control lysate (Fig. 3C, lane 3) and in lysate from arteriolar SM incubated with FN (lane 4). In contrast to Fig. 3B, phosphorylated c-Src coassociated with α_{1C} -Ca_L only in arteriolar SM incubated with FN, not in control arteriolar SM (Fig. 3C, lanes 1 and 2 vs. lanes 5 and 6, respectively). These results suggest that the association of α_{1C} -Ca_L with c-Src in

arteriolar SM is independent of FN, whereas the association of α_{1C} -Ca_L with phosphorylated c-Src depends on integrin engagement by FN.

The COOH terminus of α_{1C} -Ca_L is essential for association with β_1 -integrin. To determine whether the specific regions in the COOH terminus of α_{1C} are required for the association of α_{1C} -Ca_L with $\alpha_5\beta_1$ -integrin, three mutant α_{1C} -Ca_L constructs were overexpressed in HEK293-T cells (Fig. 1). If any of the specific regions is required for association between the two proteins, we predicted to observe less α_{1C} -Ca_L pulled down by anti- β_1 -integrin Ab in cells expressing the mutant α_{1C} -Ca_L. As shown in Fig. 4A, truncated α_{1C} -Ca_L exhibited a reduced level of association with β_1 -integrin (lane 8) and a reduced level of association with c-Src (lane 7), compared with their respective levels of association in WT α_{1C} -Ca_L (lanes 1 and 2, respectively). Figure 4B shows the same membrane after stripping and reprobing for β_1 -integrin as a loading control. The expression of β_1 -integrin, c-Src (Supplemental Fig. S3A), and the degrees of association between β_1 -integrin and c-Src were not changed in cells expressing truncated α_{1C} -Ca_L, compared with WT α_{1C} -Ca_L (quantified data not shown). When normalized to the total amount of WT α_{1C} -Ca_L, the associations between truncated α_{1C} -Ca_L and β_1 -integrin as well as between truncated α_{1C} -Ca_L and c-Src were reduced to $40 \pm 9\%$ and $55 \pm 8\%$ of WT α_{1C} -Ca_L, respectively (Fig. 4C).

Fig. 3. α_{1C} -Ca_L associates with β_1 -integrin in arteriolar smooth muscle incubated with FN. A: representative blot showing co-IP protocol on protein lysed from arteriolar smooth muscle (pulled down with anti- α_{1C} -Ca_L). β_1 -Integrin and α_{1C} -Ca_L coimmunoprecipitated when arterioles were incubated with FN for 1 h (lanes 1 and 2, respectively). Lanes 1 and 6 were immunoprecipitated with anti- α_{1C} -Ca_L from Chemicon, whereas lanes 2 and 5 were immunoprecipitated with anti- α_{1C} -Ca_L from Abcam. Lanes 3 and 4 were lysates alone isolated from control arterioles or arterioles incubated with FN, respectively. The absence of coimmunoprecipitated β_1 -integrin by rabbit IgG (lane 7) indicates binding specificity. B: representative blot showing co-IP protocol on protein lysed from arteriolar smooth muscle (pulled down with anti- α_{1C} -Ca_L). c-Src coimmunoprecipitated with α_{1C} -Ca_L whether or not arterioles were incubated with FN (lanes 1 and 2 vs. lanes 5 and 6, respectively). IP was performed as described in A. Abluminal incubation of arterioles with exogenous FN did not change the degree of association between α_{1C} -Ca_L and c-Src (lane 2, 90% and lane 5, 86% of arterioles alone, respectively). The absence of coimmunoprecipitated c-Src by rabbit IgG (lane 7) indicates binding specificity. C: representative blot showing co-IP protocol on protein lysed from arteriolar smooth muscle (pulled down with anti- α_{1C} -Ca_L). Phosphorylated c-Src was only observed in arterioles incubated with exogenous FN (lanes 1 and 2). IP was performed as described in A. The absence of phosphorylated c-Src by rabbit IgG (lane 7) indicates binding specificity.

A Arteriolar smooth muscle

B Arteriolar smooth muscle

C Arteriolar smooth muscle

A HEK293-T cells plated on fibronectin **B** HEK293-T cells plated on fibronectin **C**

Fig. 4. Requirement of the α_{1C} -Ca_L COOH terminus for association of α_{1C} -Ca_L with c-Src or β_1 -integrin. **A**: representative blot comparing WT- and truncated α_{1C} -Ca_L immunoprecipitated with either anti-c-Src, anti- β_1 -integrin Ab, or mouse IgG, then probed for α_{1C} . α_{1C} was only detected in lysates immunoprecipitated with anti-c-Src (lanes 2 and 7) or anti- β_1 -integrin Ab (lanes 1 and 8) but not with mouse IgG (lanes 3 and 6). Relatively lower amounts of α_{1C} -Ca_L were pulled down by anti-c-Src or anti- β_1 -integrin Ab in truncated α_{1C} -Ca_L (lanes 7 and 8, respectively), compared with WT. **B**: the same blot in **A** after stripping and reprobing for β_1 -integrin as a control for IP and immunoblotting. **C**: summary graph of the relative levels of immunoprecipitated α_{1C} in cells expressing WT- or truncated α_{1C} -Ca_L. Values were obtained as described in METHODS and are based on the average of at least three experiments. * $P < 0.05$ vs. WT + anti- β_1 -integrin Ab or WT + anti-c-Src Ab.

These results provide evidence for the requirement of the distal COOH terminus in the associations between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src.

To confirm that the reduced associations between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src were not due to decreased expression of truncated α_{1C} -Ca_L construct, we increased the amount of total protein for IP from cells expressing the truncated α_{1C} -Ca_L construct to twofold that of WT α_{1C} -Ca_L and reexamined the association of truncated α_{1C} -Ca_L with β_1 -integrin or with c-Src. As shown in Supplemental Fig. S2, **A** and **C**, reduced degrees of association between α_{1C} -Ca_L and β_1 -integrin (Fig. S2A, lane 3), α_{1C} -Ca_L and phosphorylated tyrosine (Tyr-Pi, Fig. S2A, lane 2), and α_{1C} -Ca_L and c-Src (Fig. S2A, lane 1) were still observed in cells expressing truncated α_{1C} -Ca_L, when compared with the degrees of their respective associations in cells expressing WT α_{1C} -Ca_L ($70 \pm 7\%$, $51 \pm 4\%$, and $58 \pm 11\%$; % of WT, respectively). However, the expressions of β_1 -integrin and c-Src, the degrees of associations between β_1 -integrin and c-Src, or between β_1 -integrin and proteins containing phosphorylated tyrosine were not changed in cells expressing truncated α_{1C} -Ca_L, compared with WT α_{1C} -Ca_L (Supplemental Figs. S2B and S3B; quantified data not shown). These results suggest a requirement for the distal COOH terminus in α_{1C} -Ca_L association with the β_1 -integrin and c-Src.

The collaborative contribution of PKA and c-Src in the association of Ca_L with β_1 -integrin. We previously showed that $\alpha_5\beta_1$ -integrin engagement leads to potentiation of Ca_L current via phosphorylation of α_{1C} -Ca_L serine (S¹⁹⁰¹) and tyrosine (Y²¹²²) by PKA and c-Src, respectively (14). Thus, a next logical step was to determine whether phosphorylation of α_{1C} -Ca_L by PKA and c-Src is required for Ca_L association with β_1 -integrin or c-Src. As shown in Fig. 5A, reduced associations between α_{1C} -Ca_L and β_1 -integrin (lane 3), α_{1C} -Ca_L and Tyr-Pi (lane 2), and α_{1C} -Ca_L and c-Src (lane 1) were observed in cells expressing S¹⁹⁰¹A/Y²¹²²F-Ca_L, compared with WT α_{1C} -Ca_L (Fig. 5C, $47 \pm 8\%$, $48 \pm 12\%$, and $27 \pm 7\%$; % of WT, respectively). However, the expressions of β_1 -integrin (Fig. 5B) and c-Src, the degrees of associations between β_1 -integrin and c-Src, or between β_1 -integrin and proteins containing phosphorylated tyrosine were unchanged in cells expressing S¹⁹⁰¹A/Y²¹²²F-Ca_L, compared with WT α_{1C} -Ca_L (Supplemental Fig. S4A, quantified data not shown). These results suggest that phosphorylation of α_{1C} -Ca_L by PKA and/or c-Src is required for the associations between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src.

The proline-rich domains in the COOH terminus are required for the association of α_{1C} -Ca_L with β_1 -integrin. Proline-rich domains (PRDs) are binding motifs for Src homology 3 domain (SH3)-containing proteins, such as c-Src (34). Two

Fig. 5. S¹⁹⁰¹ and Y²¹²² in α_{1C} -Ca_L are required for α_{1C} -Ca_L association with β_1 -integrin or c-Src. **A**: representative blot comparing WT α_{1C} -Ca_L and S¹⁹⁰¹A/Y²¹²²F α_{1C} -Ca_L immunoprecipitated with either anti-c-Src, anti-phosphorylated tyrosine (Tyr-Pi), or anti- β_1 -integrin Ab, and probed for α_{1C} . Relatively lower amounts of α_{1C} -Ca_L appear to be pulled down by anti-c-Src, anti-Tyr-Pi, or anti- β_1 -integrin Ab (lanes 1, 2, and 3, respectively) in S¹⁹⁰¹A/Y²¹²²F α_{1C} -Ca_L-expressing cells, compared with WT- α_{1C} -Ca_L. **B**: the same blot in **A** after stripping and reprobing for β_1 -integrin. **C**: summary graph showing the relative levels of immunoprecipitated α_{1C} in WT- or S¹⁹⁰¹A/Y²¹²²F- α_{1C} . Values were obtained as described in METHODS and are based on the average of at least three experiments. * P < 0.05 vs. WT + anti- β_1 -integrin Ab, WT + anti-Tyr-Pi Ab, or WT + anti-c-Src Ab.

PRDs have been identified in the COOH terminus of α_{1C} -Ca_L and have been shown to mediate the association of Ca_L with some proteins containing SH3 domains (12, 13). Thus, we investigated the roles of the COOH-terminal PRDs in determining α_{1C} -Ca_L association with β_1 -integrin or c-Src by expressing a Δ P1/ Δ P2-Ca_L mutant with selective deletion of both PRDs in the COOH terminus of α_{1C} . As shown in Fig. 6A, significantly less association between α_{1C} -Ca_L and β_1 -integrin (lane 6) and between α_{1C} -Ca_L and c-Src (lane 8), was observed in cells expressing the Δ P1/ Δ P2 α_{1C} -Ca_L mutant, compared with WT α_{1C} -Ca_L (Fig. 6C, $54 \pm 9\%$, $54 \pm 6\%$; % of WT, respectively). However, the expression of β_1 -integrin (Fig. 6B), c-Src, and the degree of association between β_1 -integrin and c-Src was unchanged in cells expressing Δ P1/ Δ P2 α_{1C} -Ca_L (Supplemental Fig. S4B, quantified data not shown). These results suggest that the PRDs in the α_{1C} -Ca_L COOH terminus are partially required for associations between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src.

Functional modulation of Ca_L current by $\alpha_5\beta_1$ -integrin is independent of the PRDs in the COOH terminus. Given the results shown in Figs. 5 and 6, we also investigated the roles

of the two PRDs in the modulation of Ca_L current following $\alpha_5\beta_1$ -integrin engagement. As shown in Fig. 7A, the application of $\alpha_5\beta_1$ -integrin antibody to the bath solution potentiated whole cell Ca_L current in HEK293-T cells expressing WT-Ca_L, consistent with our previous findings (14). To our surprise, no significant electrophysiological difference was observed between WT-Ca_L and Δ P1/ Δ P2-Ca_L (82% of WT) in the degree of current potentiation by $\alpha_5\beta_1$ -integrin engagement, suggesting that the COOH-terminal PRDs are not required for potentiation of Ca_L current by $\alpha_5\beta_1$ -integrin engagement. When the degree of Ca_L current potentiation following $\alpha_5\beta_1$ -integrin ligation in the mutant constructs was normalized to that of WT, both the truncated α_{1C} -Ca_L and S¹⁹⁰¹A/Y²¹²²F-Ca_L mutants displayed significant impairment in the amount of current potentiation by integrin ligation (Fig. 7B, 9% and 1% potentiation of WT-Ca_L, respectively; see also Ref. 14). The above results confirm that S¹⁹⁰¹ and Y²¹²² residues in the COOH terminus of α_{1C} -Ca_L are required for Ca²⁺ current potentiation following $\alpha_5\beta_1$ -integrin ligation. However, selective deletion of the two PRDs in the COOH terminus of α_{1C} -Ca_L does not impair the current potentiation by $\alpha_5\beta_1$ -integrin engagement.

A HEK293-T cells plated on fibronectin

Fig. 6. Requirement of proline-rich domains (PRDs) in the α_{1C} - Ca_L COOH terminus for α_{1C} - Ca_L association with β_1 -integrin or c-Src. **A**: representative blot comparing WT α_{1C} - Ca_L and $\Delta\text{P1}/\Delta\text{P2}$ α_{1C} - Ca_L immunoprecipitated with anti-c-Src, anti- α_5 -integrin, or anti- β_1 -integrin Ab and probed for α_{1C} . Relatively lower amounts of α_{1C} - Ca_L appear to be pulled down by anti-c-Src or anti- β_1 -integrin Ab (lanes 8 and 6, respectively) in cells expressing $\Delta\text{P1}/\Delta\text{P2}$ α_{1C} - Ca_L , compared with WT α_{1C} - Ca_L . **B**: the same blot in **A** after stripping and reprobing for β_1 -integrin. **C**: summary graph showing the relative levels of immunoprecipitated α_{1C} in WT- or $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L . Values were obtained as described in METHODS and are based on the average of at least three experiments. * $P < 0.05$ vs. WT + anti-c-Src Ab or WT + anti- β_1 -integrin Ab.

B HEK293-T cells plated on fibronectin **C**

Immunofluorescence imaging analysis demonstrates association of Ca_L with β_1 -integrin on the plasma membrane. The COOH terminus of α_{1C} - Ca_L has been shown to mediate membrane targeting of the channel (11), so we further investigated the effect of the abovementioned regions in the COOH terminus on membrane targeting of α_{1C} - Ca_L and its association with β_1 -integrin. Immunofluorescence confocal microscopy (IF) was performed to determine the expression, distribution, and relative localization of α_{1C} - Ca_L and β_1 -integrin on the plasma membrane. Images of cells without staining or cells stained with either anti-mouse IgG, anti-rabbit IgG, or the combination of both, exhibited only faint, diffuse, and nonspecific staining (Supplemental Fig. S5). As shown in Fig. 8, neither the membrane expression of α_{1C} - Ca_L nor β_1 -integrin was detectably altered in $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ - Ca_L , truncated α_{1C} - Ca_L , or $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L , compared with WT- Ca_L .

When we examined the association between WT α_{1C} - Ca_L and endogenous β_1 -integrin on the plasma membrane, a moderate degree of colocalization was observed. To quantify the degree of colocalization between α_{1C} - Ca_L and β_1 -integrin, we used the overlap of paxillin and vinculin staining as a normalization standard for the following analyses, since these two proteins highly associate at the focal adhesion site (ICA value 0.25) (37). The relatively high degree of colocalization between paxillin and vinculin was set as 100% for subsequent

analysis of colocalization between α_{1C} - Ca_L and β_1 -integrin. We used Pearson's coefficient (R), Mander's coefficient (R_T), and ICA to determine the degree of α_{1C} - Ca_L - β_1 -integrin colocalization, as listed in Table 1. The degrees of α_{1C} - Ca_L colocalization with β_1 -integrin in WT- Ca_L , truncated α_{1C} - Ca_L , $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ - Ca_L , or $\Delta\text{P1}/\Delta\text{P2}$ α_{1C} - Ca_L were significantly less than the degree of colocalization between paxillin and vinculin (60–88% of paxillin-vinculin colocalization).

Using ICA analysis (Fig. 9), modest, but not significant, decreases in the degrees of α_{1C} - Ca_L association with β_1 -integrin were observed in the truncated α_{1C} - Ca_L , $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ α_{1C} - Ca_L , and $\Delta\text{P1}/\Delta\text{P2}$ α_{1C} - Ca_L mutants, compared with WT- Ca_L ($99 \pm 5.9\%$, $99 \pm 6.2\%$, and $88 \pm 5.1\%$ of WT α_{1C} - Ca_L , respectively). Deconvolution analysis was also used to eliminate the signals collected from out-of-focus emission. However, no substantial differences were noted in the same comparisons when deconvolution analysis was applied (data not shown). Collectively, confocal image analysis demonstrated that the association between α_{1C} - Ca_L and β_1 -integrin on the cell membrane was moderate in cells expressing WT α_{1C} - Ca_L . No significant differences were observed in the degree of α_{1C} - Ca_L colocalization with β_1 -integrin between the three mutant α_{1C} - Ca_L constructs, compared with WT α_{1C} - Ca_L . Taken together, the IF results suggest that α_{1C} - Ca_L and β_1 -integrin colocalize to a moderate degree on the plasma mem-

Fig. 7. Electrophysiological protocols to determine the effects of COOH terminus-specific regions on modulation of Ca_L current by $\alpha_5\beta_1$ -integrin. **A**: representative recordings of whole cell Ca_L current from HEK293-T cells expressing WT- Ca_L (left) or $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L (right). Top traces in both panels show the amount of basal current in cells expressing WT- or $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L , and bottom traces show the current potentiation by $\alpha_5\beta_1$ -integrin Ab (10 $\mu\text{g}/\text{ml}$). No substantial difference in the relative amount of Ca_L current potentiation was observed in cells expressing $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L , compared with WT- Ca_L . **B**: summary graph showing peak current potentiation by $\alpha_5\beta_1$ -integrin in WT- Ca_L , truncated α_{1C} - Ca_L , or $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L . Ca_L current potentiated by $\alpha_5\beta_1$ -integrin Ab was significantly attenuated in truncated α_{1C} - Ca_L or in $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ - Ca_L , but not in $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L . Number of cells recorded is as follows: WT- Ca_L , $n = 18$; $n = 8$ in cells expressing $\Delta\text{P1}/\Delta\text{P2}$ - Ca_L , $\text{S}^{1901}\text{A}/\text{Y}^{2122}\text{F}$ - Ca_L , or truncated α_{1C} - Ca_L . * $P < 0.05$ vs. WT- Ca_L .

brane and that the modifications we made in the COOH terminus of α_{1C} - Ca_L did not significantly alter the membrane targeting of α_{1C} - Ca_L or β_1 -integrin on the plasma membrane. Thus, the reduced associations between the mutant forms of α_{1C} - Ca_L and β_1 -integrin observed in IP protocol are not due to deficient membrane targeting.

DISCUSSION

We present evidence that the pore-forming α_{1C} -subunit of the L-type calcium channel (α_{1C} - Ca_L) associates with β_1 -integrin and c-Src in arteriolar SM as well as when the channel is heterologously expressed in HEK293-T cells. These associations depend on interactions of the cells with the ECM protein FN, which is known from previous studies to potentiate Ca_L current following $\alpha_5\beta_1$ -integrin engagement (40). Furthermore, our results show that the associations between α_{1C} - Ca_L and $\alpha_5\beta_1$ -integrin and between α_{1C} - Ca_L and c-Src require specific regions in the COOH terminus of α_{1C} - Ca_L . Two specific domains that are critical for these interactions are the PKA and c-Src phosphorylation sites that we previously identified as essential for modulation of Ca_L function following $\alpha_5\beta_1$ -integrin engagement (14). In addition, we found that Ca_L preferentially associates with the phosphorylated, active, form of c-Src in arteriolar SM and that this association depends on FN. The latter result provides evidence for a unique local functional regulation of Ca_L by $\alpha_5\beta_1$ -integrin through phosphorylation of c-Src. Collectively, our data support the concept

of a macromolecular complex composed of multiple focal adhesion molecules including PKA, c-Src, and some fraction of the Ca_L population, which is assembled upon integrin ligation and required for potentiation of Ca_L current following $\alpha_5\beta_1$ -integrin engagement.

In this study, three different approaches, IP, IF, and patch-clamp recording of Ca_L current, were used to assess structural and functional interactions between α_{1C} - Ca_L and $\alpha_5\beta_1$ -integrin. In some respects, the data obtained using these methods support each other, while in other respects the data conflict. How can we reconcile the conflicting results? What do the collective data tell us about the direct or indirect association of the channel with $\alpha_5\beta_1$ -integrin? What signaling components are required to mediate α_{1C} - Ca_L association with $\alpha_5\beta_1$ -integrin upon adhesion to FN? How does the spatial interaction between α_{1C} - Ca_L and $\alpha_5\beta_1$ -integrin contribute to the functional regulation of Ca_L ? What is the physiological significance of α_{1C} - Ca_L association with $\alpha_5\beta_1$ -integrin? These issues will be addressed in the following sections.

Direct or indirect association of Ca_L with $\alpha_5\beta_1$ -integrin. After demonstrating an association between α_{1C} - Ca_L and $\alpha_5\beta_1$ -integrin, using IP and IB methods, we sought to determine the nature of the interaction and the regions in α_{1C} - Ca_L that were required. Because the crystal structure of α_{1C} - Ca_L has not been resolved, interactions between these proteins are difficult to predict. If α_{1C} - Ca_L directly interacts with $\alpha_5\beta_1$ -integrin, a potential binding region would be the canonical

Fig. 8. Confocal immunofluorescence image analysis to determine the distribution and association of α_{1C} -Ca_L and β_1 -integrin on the plasma membrane. Representative confocal immunofluorescence images from the cells expressing WT-, truncated α_{1C} -, S^{1901A/Y2122F}-, or $\Delta P1/\Delta P2$ -Ca_L. α_{1C} and endogenous β_1 -integrin expression on the plasma membrane appear as green and red punctate staining in *a*, *e*, *i*, and *m* and in *b*, *f*, *j*, and *n*, respectively. The colocalization of α_{1C} -Ca_L with β_1 -integrin on the membrane appears as yellow to orange punctate staining (*c*, *g*, *k*, and *o*). A noticeably higher degree of α_{1C} -Ca_L colocalization with β_1 -integrin was seen in cells expressing WT- α_{1C} -Ca_L, compared with mutant α_{1C} -Ca_L construct-expressing cells. Merged images of the previous three panels with nuclei counterstaining appear in blue (*d*, *h*, *l*, and *p*). Endogenous paxillin and vinculin staining in HEK293-T cells appears as green and red in *q* and *r*, respectively. The degree of colocalization between paxillin and vinculin (yellow to orange staining; *s*) appears to be much higher than that between α_{1C} -Ca_L and β_1 -integrin in the WT- or mutant α_{1C} -Ca_L-expressing cells. Merged image with nuclear counterstaining in blue appears in *t*. Magnification, $\times 63$ oil; scale bar, 10 μ m.

Arg-Gly-Asp (RGD)-integrin-binding motif. In fact, a previous study by McPhee et al. (31) identified the functional significance of an RGD sequence located in the extracellular loop, between the first membrane-spanning region and the pore, of the G protein-activated inward rectifier K⁺ channel (GIRK). This RGD sequence mediates the direct interaction between GIRK and the β_1 -integrin. The functional significance of the interaction appears to involve targeting of GIRK to the plasma membrane and/or regulation of its stability rather than acute regulation of channel function (31). However, in Cav1.2, the

only conserved RGD sequence (aa 448–450) is found in a conserved transmembrane region of all three Ca_L isoforms, so that direct binding of α_{1C} -Ca_L to RGD-binding integrins is unlikely due to potential steric hindrance. Thus, an indirect association between α_{1C} -Ca_L and $\alpha_5\beta_1$ -integrin through cytoplasmic or cytoskeletal proteins seems more probable.

To determine regions in α_{1C} -Ca_L that provide binding motifs for other focal adhesion proteins that are known to associate with $\alpha_5\beta_1$ -integrin, we used a site-directed mutagenesis strategy. On the basis of differences in the amount of coimmuno-

Table 1. *Semiquantitative analysis of Ca_L association with β_1 -integrin*

	R_r , % of Pax + Vin	R_r , % of Pax + Vin	ICA, %	ICA, % of Pax + Vin
Paxillin-vinculin	100	100	0.251 ± 0.021	100
WT	60.6 ± 2.7	88.5 ± 1.5	0.22 ± 0.009	85.1 ± 4.2
Truncated- α_{1C}	59.2 ± 4.8	87.9 ± 2.6	0.211 ± 0.01	84.1 ± 5.0
$S^{1901}A/Y^{2122}F$	56.5 ± 4.5	84.5 ± 2.5	0.21 ± 0.011	84.8 ± 5.3
$\Delta P1/\Delta P2$	55.4 ± 5.1	84.1 ± 1.7	0.185 ± 0.013	75.2 ± 7.4

Values are means $\pm$ SE, taken from at least eight cells in either wild-type (WT) or mutant L-type voltage-gated Cav1.2 calcium channel (Ca_L) per experiment. The degree of Ca_L association with β_1 -integrin was normalized using the association of paxillin (Pax) and vinculin (Vin) as a reference and set as 100% for Pearson's, Mander's, or intensity correlation analysis (ICA). The value of Ca_L association with β_1 -integrin in WT, truncated α_{1C} , $S^{1901}A/Y^{2122}F$, or $\Delta P1/\Delta P2$ - Ca_L is presented as the percentage $\pm$ SE of paxillin-vinculin association. R_r , Pearson's coefficient; R , Mander's coefficient. Statistical analysis was performed on the basis of the average from at least four individual experiments.

precipitated α_{1C} - Ca_L with β_1 -integrin in WT versus mutated α_{1C} - Ca_L , we identified several regions in the α_{1C} - Ca_L COOH terminus that are involved in the interaction of α_{1C} - Ca_L with $\alpha_5\beta_1$ -integrin: two phosphorylation sites for PKA and c-Src, along with the PRDs. These findings therefore implicate PKA, c-Src, and potentially other SH3 domain-containing proteins in mediating the interaction of α_{1C} - Ca_L with $\alpha_5\beta_1$ -integrin.

The roles of PKA in the functional regulation of Ca_L and its association with $\alpha_5\beta_1$ -integrin. PKA is known to regulate all three Ca_L isoforms. In neurons, PKA colocalizes with adenylylate cyclase, β_2 -adrenergic receptor (β_2 -AR), A-kinase anchoring protein (AKAP), and protein phosphatase (PP2A) in a macromolecular signaling complex; PKA mediates the increase in neuronal Ca_L current following stimulation of β_2 -AR (6). Our results suggest that PKA is also a component of another macromolecular complex containing various focal adhesion proteins recruited following ligation of $\alpha_5\beta_1$ -integrin and that signaling between proteins in this complex also can exert a significant degree of control over Ca_L function. Evidence to support this conclusion derives from two aspects of our results. First, reduced association between α_{1C} - Ca_L and β_1 -integrin was observed in an α_{1C} - Ca_L mutant with altered PKA and c-Src phosphorylation sites. Second, our previous

electrophysiological studies demonstrated a substantial reduction in the amount of Ca_L current that was potentiated following ligation of $\alpha_5\beta_1$ -integrin when α_{1C} - Ca_L mutants with altered PKA and/or c-Src phosphorylation were used (14). When S^{1901} in α_{1C} is mutated, it not only leads to reduction in the association between α_{1C} - Ca_L and β_1 -integrin, but also to reduction in the phosphorylation of Ca_L by PKA (14). Findings from other laboratories also support the notion that S^{1901} in the COOH terminus of α_{1C} - Ca_L is a focal assembly point for PKA, AKAP, PP2A, and WAVE/WASP, which collectively modulate Ca_L function (5). In support of this idea, the macromolecular complex composed of PKA, AKAP, PP2A, and WAVE/WASP has been shown to provide a spatial link to the integrin-cytoskeleton network in modulation of other cellular functions (15) and engagement of β_1 -integrin is known to facilitate PKA targeting to specific cellular locations (26, 32). On the basis of all the above, faulty trafficking/targeting of PKA in the cytoplasm could contribute to reduced interaction of PKA with other focal adhesion components in the macromolecular complex, thereby disrupting the spatial link to the integrin-cytoskeleton network and reducing the extent to which Ca_L function is influenced by $\alpha_5\beta_1$ -integrin signaling.

The roles of PRDs and c-Src in functional regulation of Ca_L and its association with $\alpha_5\beta_1$ -integrin. c-Src is another kinase that is implicated in the signaling pathway between $\alpha_5\beta_1$ -integrin and Ca_L . The involvement of c-Src is supported by several findings in the present study: 1) co-IP of α_{1C} - Ca_L with c-Src in arteriolar SM appears to be independent of FN; 2) co-IP of α_{1C} - Ca_L and phosphorylated c-Src is only observed in arteriolar SM incubated with FN; 3) co-IP of α_{1C} - Ca_L and c-Src is only observed upon adhesion of HEK293-T cells to FN; and 4) mutation of the phosphorylation site for c-Src in the COOH terminus in α_{1C} - Ca_L (Y^{2122}) leads to reduced co-IP of α_{1C} - Ca_L with β_1 -integrin and of α_{1C} - Ca_L with c-Src. These results are consistent with previous findings that purified c-Src associates with the COOH terminus of α_{1C} - Ca_L (19, 20) and that a basal level of Ca_L current depends on c-Src activity in colonic smooth muscle (16). Our co-IP findings are also consistent with a previous functional study from our laboratory showing that the potentiation of Ca_L current following $\alpha_5\beta_1$ -integrin ligation is significantly reduced in $Y^{2122}F$ α_{1C} - Ca_L constructs (14). Thus, preventing this tyrosine residue from being phosphorylated not only reduces the overall phosphorylation of Ca_L by exogenous c-Src, but also reduces the spatial interaction between α_{1C} - Ca_L and β_1 -integrin and between α_{1C} - Ca_L and endogenous c-Src, indicating that the interactions between α_{1C} - Ca_L and β_1 -integrin and between α_{1C} - Ca_L and

Fig. 9. Bar graph showing the quantified degree of α_{1C} - Ca_L association with β_1 -integrin normalized to the degree of paxillin (Pax)-vinculin (Vin) association using intensity correlation analysis (ICA). The association of paxillin with vinculin was set as 100%. The ICA value of α_{1C} - Ca_L - β_1 -integrin association in WT α_{1C} - Ca_L , truncated α_{1C} - Ca_L , $S^{1901}A/Y^{2122}F$ - α_{1C} - Ca_L , or $\Delta P1/\Delta P2$ α_{1C} - Ca_L is presented as the percentage of paxillin-vinculin association. Values were taken from at least 8 cells in either WT- or mutant Ca_L per experiment, and the values in the graph were calculated on the basis of the average of at least four experiments. Statistical analysis was performed on the basis of the average from at least four individual experiments. # $P < 0.01$ vs. the degree of colocalization between paxillin and vinculin.

c-Src are required for current potentiation following $\alpha_5\beta_1$ -integrin ligation.

Our IP studies also identified a region in the α_{1C} -Ca_L COOH terminus containing two PRDs (P1/P2) as another potential binding domain for c-Src. Reduced associations between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src were observed in the $\Delta P1/\Delta P2$ α_{1C} -Ca_L mutant. Our results agree with a previous study demonstrating that fusion proteins containing α_{1C} PRDs mediate interactions with c-Src in SY5Y cells expressing the neuronal Ca_L isoform upon IGF stimulation (1). Taken together, the results suggest multiple roles for PRDs in association with the macromolecular complex containing β_1 -integrin, c-Src, and α_{1C} -Ca_L.

FAK is another proline-rich tyrosine kinase known to provide a binding site for SH2 and SH3 domain-containing proteins such as paxillin, vinculin, talin, p130Cas, and Crk (42). Although we did not specifically examine whether α_{1C} -Ca_L associates with FAK, our previous studies in vascular SMC revealed that a significant amount of the Ca_L current potentiation following $\alpha_5\beta_1$ -integrin engagement was attenuated by dialysis of the cells with an antibody against FAK (40). A precedent for an association between FAK and another ion channel has been established by studies of Cherubini et al. (4), who demonstrated the association between FAK and the human *ether-a-go-go*-related gene (hERG) channel. In addition, PYK2, a family member of the FAK tyrosine kinases, was demonstrated to associate with cardiac α_{1C} -Ca_L via binding to PRDs (9). On the basis of the above, the PRDs in the α_{1C} -Ca_L appear to play significant roles in the assembly of a macromolecular complex containing Ca_L, $\alpha_5\beta_1$ -integrin, c-Src, and potentially other protein tyrosine kinases.

It should be noted that the roles of PRDs in the physical and functional association of α_{1C} -Ca_L with $\alpha_5\beta_1$ -integrin may be more complicated than at first suggested by the preceding discussion. When the $\Delta P1/\Delta P2$ α_{1C} -Ca_L mutant is expressed, the IP results (Fig. 6, A and C) reveal a reduced association between α_{1C} -Ca_L and β_1 -integrin and between α_{1C} -Ca_L and c-Src, compared with WT α_{1C} -Ca_L, whereas the electrophysiological data (Fig. 7, A and B) reveal no substantial impairment in the degree of Ca_L current potentiation following $\alpha_5\beta_1$ -integrin ligation. The discrepancy may arise from two issues. First, the PRD regions that we deleted from α_{1C} -Ca_L lie within the distal COOH terminus inhibitory region (DCT). Two studies have shown that deletion of the homologous PRDs (aa 1966–2004) in the cardiac Ca_L isoform leads to enhancement of basal Ca_L current (13, 38). Deletion of the PRDs removes part of the DCT, which may partially relieve constitutive channel inhibition by the DCT segment, though this has not been specifically tested. However, the major differences between the neuronal, cardiac, and smooth muscle isoforms of the Ca_L channel reside in the more distal regions of the respective COOH termini, and whether or not the DCT segment exerts strong inhibitory control of the neuronal Ca_L has not been determined.

A second explanation for the discrepancy between the structural and functional PRD results is the possible contribution of another PRD that has been identified in the II–III linker region of α_{1C} -Ca_L (aa 857–861). A study by Dubuis et al. (9) suggests that the II–III linker region appears to play a role in extending the activation range of cardiac Ca_L, which consequently alters Ca_L function. Our IP results revealed that deletion of the

COOH-terminal PRDs only reduced c-Src coassociation with α_{1C} -Ca_L by 42%, even when the amount of truncated COOH-terminal α_{1C} -Ca_L mutants for IP was increased by twofold (Supplemental Fig. S2, A and C). Thus, deletion of only the PRDs in the COOH terminus of α_{1C} -Ca_L may not be sufficient to fully abolish the potentiation in Ca_L current following $\alpha_5\beta_1$ -integrin engagement. Overall, our electrophysiological and IP results agree with the findings of Dubuis et al. and suggest the possibility of another important c-Src binding region in α_{1C} -Ca_L.

Reduced association between α_{1C} -Ca_L and β_1 -integrin is not due to faulty membrane targeting of Ca_L. The amino acid residues 1623–1733 in the COOH terminus of cardiac α_{1C} -Ca_L have been shown to mediate the membrane targeting of the channel (11). Using IF, we detected no significant difference in membrane targeting of α_{1C} -Ca_L or β_1 -integrin in S¹⁹⁰¹A/Y²¹²²F-Ca_L, truncated α_{1C} -Ca_L at amino acid residue 1862, or $\Delta P1/\Delta P2$ -Ca_L, compared with WT-Ca_L. Our results suggest that the reduced association between α_{1C} -Ca_L and β_1 -integrin observed in the above mentioned α_{1C} -Ca_L mutants is not due to unsuccessful membrane targeting of α_{1C} -Ca_L. Our findings agree with those of Gao et al. (11) that the regions in the COOH terminus required for successful membrane targeting of cardiac Ca_L are amino acid residues 1623–1733. Because those specific regions are not altered in our mutant α_{1C} -Ca_L constructs, the reduced association between α_{1C} -Ca_L and β_1 -integrin in our mutant constructs are unlikely attributable to incorrect membrane targeting of Ca_L. In addition, our IF results also suggest that the association between α_{1C} -Ca_L and β_1 -integrin on the plasma membrane is independent of the PKA/c-Src phosphorylation sites, the PRD domains, or the distal COOH terminus after amino acid residue 1862 in α_{1C} -Ca_L. These results suggest that $\alpha_5\beta_1$ -integrin modulates Ca_L function independent of the interactions of the two proteins on the plasma membrane. The IF results agree with the IP and patch-clamp findings in that the formation of a macromolecular complex including PKA, c-Src, $\alpha_5\beta_1$ -integrin, and Ca_L in the cytoplasm following $\alpha_5\beta_1$ -integrin engagement appears to play an important role in Ca_L current potentiation by $\alpha_5\beta_1$ -integrin.

Physiological significance of Ca_L association with integrin. Our findings of an association between Ca_L and β_1 -integrin and between Ca_L and c-Src provide evidence for a macromolecular signaling complex composed of $\alpha_5\beta_1$ -integrin, Ca_L, c-Src, PKA, and possibly other focal adhesion components. Formation of this complex appears to be important for regulation of Ca_L function following integrin-ECM interaction. Our IP results are consistent with previous findings that c-Src is involved in the increase of Ca_L current potentiation in response to PDGF (17) or IGF (1) and with the finding that a c-Src-FAK complex forms in colonic smooth muscle in response to PDGF stimulation (16). Interestingly, cross talk between integrins and growth factors has been demonstrated in several studies, with the interaction of FAK and c-Src as a point of convergence (10, 28, 35). Although it is not known whether these two signaling pathways converge to regulate an ion channel, an intriguing possibility is that PDGF or IGF act in conjunction with $\alpha_5\beta_1$ -integrin to produce additive or synergistic potentiation of Ca_L function by integrin.

In summary, this is the first study to provide evidence for the association of α_{1C} -Ca_L with $\alpha_5\beta_1$ -integrin or α_{1C} -Ca_L with c-Src in arteriolar smooth muscle. This association depends on

engagement of $\alpha_5\beta_1$ -integrin by FN and specific regions in the α_{1C} -Ca_L COOH terminus, including two phosphorylation sites for PKA and c-Src and proline-rich domains that provide a binding motif for SH3 domain-containing proteins, such as c-Src. Identification of these regions, along with our electrophysiological findings, suggests that a macromolecular complex is assembled upon engagement of $\alpha_5\beta_1$ -integrin and that the interactions of proteins within this complex contribute to modulation of Ca_L function. Our study provides new insights into a Ca_L macromolecular regulatory complex that is required for functional regulation of the channel following $\alpha_5\beta_1$ -integrin engagement.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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