Spatial association of the Cav1.2 calcium channel with α5β1-integrin

Jun-Tzu Chao,1 Peichun Gui,1 Gerald W. Zamponi,2 George E. Davis,1 and Michael J. Davis1

1Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri; and 2Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada

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Chao J, Gui P, Zamponi GW, Davis GE, Davis MJ. Spatial association of the Cav1.2 calcium channel with α5β1-integrin. Am J Physiol Cell Physiol 300: C477–C489, 2011.First published December 22, 2010; doi:10.1152/ajpcell.00171.2010.—Engagement of α5β1-integrin by fibronectin (FN) acutely enhances Cav1.2 channel (Cav1.2) current in rat arteriolar smooth muscle and human embryonic kidney cells (HEK293-T) expressing Cav1.2. Using coimmunoprecipitation strategies, we show that coassociation of Cav1.2 with α5- or β1-integrin in HEK293-T cells is specific and depends on cell adhesion to FN. In rat arteriolar smooth muscle, coassociations between Cav1.2 and α5β1-integrin and between Cav1.2 and phosphorylated c-Src are also revealed and enhanced by FN treatment. Using site-directed mutagenesis of Cav1.2 heterologously expressed in HEK293-T cells, we identified two regions of Cav1.2 required for these interactions: 1) COOH-terminal residues Ser1901 and Tyr2112, 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 Cav1.2 colocalization with β1-integrin on the plasma membrane. Collectively, our results strongly suggest that 1) upon ligation by FN, Cav1.2 associates with α5β1-integrin in a macromolecular complex including PKA, c-Src, and potentially other protein kinases; 2) phosphorylation of Cav1.2 at Y2112 and/or S1901 is required for association of Cav1.2 with α5β1-integrin; and 3) c-Src, via binding to PRDs that reside in the II–III linker region and/or the COOH terminus of Cav1.2, mediates current potentiation following α5β1-integrin engagement. These findings provide new evidence for how interactions between α5β1-integrin and FN can modulate Cav1.2 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. Ca2+ entry through the L-type voltage-gated Cav1.2 calcium channel (Cav1.2) is required for stretch-induced contraction of vascular smooth muscle (VSM). Intracellular pressurization of isolated arterial myocytes leads to enhancement of Cav1.2 current, suggesting that Cav1.2 may be modulated by mechanical stress applied to the plasma membrane (22). A link between Cav1.2 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 α5-, β1-, and β3-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 Ca2+ entry through Cav1.2 because electrophysiological studies reveal selective effects of integrin ligands on Cav1.2 current; the engagement and clustering of α5β1-integrin by insoluble fibronectin (FN) or anti-α5-integrin antibody acutely potentiates Cav1.2 current, whereas engagement of α5β1-integrin by vitronectin or soluble ligands inhibits Cav1.2 current in isolated VSM cells (41). Using site-directed mutagenesis strategies, two phosphorylation sites within the COOH terminus of the αC5-subunit of Cav1.2 (αC5-Cav1.2) were identified as targets of protein kinase A (PKA) and c-Src that become activated downstream from α5β1-integrin ligation (14).

A critical remaining issue is the extent to which α5β1-integrin, PKA, c-Src, and Cav1.2 are spatially coupled within a cell. In the present study, we tested the hypothesis that α5β1-integrins spatially associate with Cav1.2 and that this association is required to modulate Cav1.2 function. Using human embryonic kidney cells (HEK293-T) overexpressing the neuronal isoform of Cav1.2 and VSM cells from rat skeletal muscle arterioles as model systems, the interactions between endogenous α5β1-integrin and Cav1.2 and between c-Src and Cav1.2 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% CO2 at 37°C. When cells reached 70–80% confluence, wild-type (WT) or mutant neuronal Cav1.2 calcium channel (Cav1.2) cDNAs composed of αC5-C (6 μg), β1B (3 μg), and α5β2 (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 Cav1.2 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 αC5-Cav1.2 due to
variation in transfection efficiency among the different CaL mutants, the experiments for all CaL mutants were performed in parallel with WT-CaL 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 α1C–COOH terminus were generated using site-directed mutagenesis as described previously (21, 40, 41), except for addition of 1× 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 MgCl2, 10 HEPES, and 1 CaCl2 (pH 7.2 with CsOH). Ba2+ 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 (in mM) 110 CsCl, 20 TEA chloride, 10 EGTA, 2 mM MgCl2, 10 HEPES, and 1 CaCl2 (pH 7.2 with CsOH). Ba2+ 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.

Immunofluorescence confocal imaging. To determine the distribution and expression of α1C-CaL and β1-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). α1C-CaL 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). β1-Integrin was detected using mouse anti-human β1-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. A21206, Invitrogen). Counterstaining of nuclei was performed using TO-PRO-3 (0.5 μM, catalog no. T3605, Invitrogen). 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 α1C-CaL to β1-integrin in WT-α1C-CaL-expressing cells was set as 1. The ratio of immunoprecipitated α1C-CaL to β1-integrin in mutant α1C-CaL-expressing cells was expressed relative to WT-α1C-CaL.
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 ×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 μm and a zoom factor of 5. The image size was ~49.2 μm².

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 α1C-CaL-subunit; 543/569 nm (excitation/emission) for β1-integrin, and 633/656 nm (excitation/emission) for nuclear staining.

Quantification of α1C-CaL association with β1-integrin. The association of α1C-CaL with β1-integrin was analyzed with ImageJ software (version 1.39d, National Institutes of Health, Bethesda, MD). The degree of α1C-CaL association with β1-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 coincidently (24). ICA values are distributed between −0.5 and +0.5. ICA values close to 0 indicate random staining, values −0.5 ≤ ICA < 0 represent segregated staining, and values 0 < ICA ≤ +0.5 represent interdependent staining. The degree of α1C-CaL association with β1-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 ± SE from at least three independent experiments for four independent experiments for immunofluorescence confocal imaging (IP). 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**

CaL association with α5β1-integrin depends on adhesion to fibronectin. To determine whether α1C-CaL associated with α5β1-integrin, IP of whole cell lysates from HEK293-T cells expressing the neuronal CaL using either anti-β1- or anti-α5-integrin antibody was followed by IB to probe for the presence of α1C-CaL. The neuronal CaL isoform was used as a model system because α1C-CaL mutants had been previously generated by site-directed mutagenesis strategies; neuronal and smooth muscle (SM) cell (SMC) CaL 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 α5β1-integrin ligation (14). We first determined the association between α1C-CaL and β1-integrin in cells plated on poly-L-lysine-treated dishes. As shown in Fig. 2A, no appreciable α1C-CaL was observed after IP using anti-β1-integrin Ab in either control (untransfected, lane 2) or cells expressing WT α1C-CaL (lane 7) when plated on poly-L-lysine-treated dishes. However, when cells expressing WT α1C-CaL were plated on FN (Fig. 2B), α1C-CaL was detected after IP of the lysate using either anti-α5- (lane 4) or anti-β1-integrin antibody (lane 3). No specific α1C-CaL band was observed in cells expressing α1C-CaL plated on BSA-coated dishes after IP using anti-β1-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 α1C-CaL with α5 or β1-integrin in HEK293-T cells is specific and depends on cell adhesion to FN.

To confirm that the effect of FN on α1C-CaL association with β1-integrin was not an artifact of CaL overexpression in HEK293-T cells, we examined the same interaction in lysates from arteriolar SM. Arterioles were dissected and then incubated with exogenous FN abluminally (300 μg/ml) for 1 h. When arteriolar SM lysates were immunoprecipitated with...
anti-α1C-CaL β1-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 α1C-CaL. In addition, incubation with FN did not substantially change the level of α1C-CaL expression in arterioles (Supplemental Fig. S1B). These results indicate that the association of α1C-CaL with β1-integrin in SMC depends on ligation of β1-integrin by FN.

c-Src, a tyrosine kinase activated upon integrin ligation by ECM ligands (18), has been shown to modulate CaL function in SMC (2, 3, 19, 39). Thus, we also examined whether c-Src associated with α1C-CaL 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-α1C-CaL antibodies (Fig. 3B, lanes 1 and 2). However, abluminal incubation with FN did not substantially change the degree of c-Src association with α1C-CaL. 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 α1C-CaL only in arteriolar SM incubated with FN, as not in control arteriolar SM (Fig. 3C, lanes 1 vs. lanes 5 and 6, respectively). These results suggest that the association of α1C-CaL with c-Src in arteriole SM is independent of FN, whereas the association of α1C-CaL with phosphorylated c-Src depends on integrin engagement by FN.

The COOH terminus of α1C-CaL is essential for association with β1-integrin. To determine whether the specific regions in the COOH terminus of α1C-CaL are required for the association of α1C-CaL with αβ1-integrin, three mutant α1C-CaL 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 α1C-CaL pulled down by anti-β1-integrin Ab in cells expressing the mutant α1C-CaL. As shown in Fig. 4A, truncated α1C-CaL exhibited a reduced level of association with β1-integrin (lane 8) and a reduced level of association with c-Src (lane 7), compared with their respective levels of association in WT α1C-CaL (lanes 1 and 2, respectively). Figure 4B shows the same membrane after stripping and reprobing for β1-integrin as a loading control. The expression of β1-integrin, c-Src (Supplemental Fig. S3A), and the degrees of association between β1-integrin and c-Src were not changed in cells expressing truncated α1C-CaL, compared with WT α1C-CaL (quantified data not shown). When normalized to the total amount of WT α1C-CaL, the associations between truncated α1C-CaL and β1-integrin as well as between truncated α1C-CaL and c-Src were reduced to 40 ± 9% and 55 ± 8% of WT α1C-CaL, respectively (Fig. 4C).
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These results provide evidence for the requirement of the 
distal COOH terminus in the associations between \(\alpha_{1C}-\text{CaL}\) and \(\beta_1\)-integrin and between \(\alpha_{1C}-\text{CaL}\) and c-Src.

To confirm that the reduced associations between \(\alpha_{1C}-\text{CaL}\) and \(\beta_1\)-integrin and between \(\alpha_{1C}-\text{CaL}\) and c-Src were not due to decreased expression of truncated \(\alpha_{1C}-\text{CaL}\), we increased the amount of total protein for IP from cells expressing the truncated \(\alpha_{1C}-\text{CaL}\) construct to twofold that of WT \(\alpha_{1C}-\text{CaL}\) and reexamined the association of truncated \(\alpha_{1C}-\text{CaL}\) with \(\beta_1\)-integrin or with c-Src. As shown in Supplemental Fig. S2, A and C, reduced degrees of association between \(\alpha_{1C}-\text{CaL}\) and \(\beta_1\)-integrin (Fig. S2A, lane 3), \(\alpha_{1C}-\text{CaL}\) and phosphorylated tyrosine (Tyr-Pi, Fig. S2A, lane 2), and \(\alpha_{1C}-\text{CaL}\) and c-Src (Fig. S2A, lane 1) were still observed in cells expressing truncated \(\alpha_{1C}-\text{CaL}\), when compared with the degrees of their respective associations in cells expressing WT \(\alpha_{1C}-\text{CaL}\) (70 ± 7%, 51 ± 4%, and 58 ± 11%; % of WT, respectively). However, the expressions of \(\beta_1\)-integrin and c-Src, the degrees of associations between \(\beta_1\)-integrin and c-Src, or between \(\beta_1\)-integrin and proteins containing phosphorylated tyrosine were not changed in cells expressing truncated \(\alpha_{1C}-\text{CaL}\), compared with WT \(\alpha_{1C}-\text{CaL}\) (Supplemental Figs. S2B and S3B; quantified data not shown). These results suggest a requirement for the distal COOH terminus in \(\alpha_{1C}-\text{CaL}\) association with the \(\beta_1\)-integrin and c-Src.

The collaborative contribution of PKA and c-Src in the association of \(\text{CaL}\) with \(\beta_1\)-integrin. We previously showed that \(\alpha_\beta_{1}\)-integrin engagement leads to potentiating of \(\text{CaL}\) current via phosphorylation of \(\text{CaL}\) serine (S\(^{1901}\)) and tyrosine (Y\(^{2122}\)) by PKA and c-Src, respectively (14). Thus, a next logical step was to determine whether phosphorylation of \(\alpha_{1C}-\text{CaL}\) by PKA and c-Src is required for \(\text{CaL}\) association with \(\beta_1\)-integrin or c-Src. As shown in Fig. 5A, reduced associations between \(\alpha_{1C}-\text{CaL}\) and \(\beta_1\)-integrin (lane 3), \(\alpha_{1C}-\text{CaL}\) and Tyr-Pi (lane 2), and \(\alpha_{1C}-\text{CaL}\) and c-Src (lane 1) were observed in cells expressing S\(^{1901}\)A/Y\(^{2122}\)F-\(\text{CaL}\), compared with WT \(\alpha_{1C}-\text{CaL}\) (Fig. 5C, 47 ± 8%, 48 ± 12%, and 27 ± 7%; % of WT, respectively). However, the expressions of \(\beta_1\)-integrin (Fig. 5B) and c-Src, the degrees of associations between \(\beta_1\)-integrin and c-Src, or between \(\beta_1\)-integrin and proteins containing phosphorylated tyrosine were unchanged in cells expressing S\(^{1901}\)A/Y\(^{2122}\)F-\(\text{CaL}\), compared with WT \(\alpha_{1C}-\text{CaL}\) (Supplemental Fig. S4A; quantified data not shown). These results suggest that phosphorylation of \(\alpha_{1C}-\text{CaL}\) by PKA and/or c-Src is required for the associations between \(\alpha_{1C}-\text{CaL}\) and \(\beta_1\)-integrin and between \(\alpha_{1C}-\text{CaL}\) and c-Src.

The proline-rich domains in the COOH terminus are required for the association of \(\alpha_{1C}-\text{CaL}\) with \(\beta_1\)-integrin. Proline-rich domains (PRDs) are binding motifs for Src homology 3 domain (SH3)-containing proteins, such as c-Src (34). Two
PRDs have been identified in the COOH terminus of α1C-CaL and have been shown to mediate the association of CaL with some proteins containing SH3 domains (12, 13). Thus, we investigated the roles of the COOH-terminal PRDs in determining α1C-CaL association with β1-integrin or c-Src by expressing a P1/P2-CaL mutant with selective deletion of both PRDs in the COOH terminus of α1C. As shown in Fig. 6A, significantly less association between α1C-CaL and β1-integrin (lane 6) and between α1C-CaL and c-Src (lane 8), was observed in cells expressing the ΔP1/ΔP2 α1C-CaL mutant, compared with WT α1C-CaL (Fig. 6C, 54 ± 9%, 54 ± 6%; % of WT, respectively). However, the expression of β1-integrin (Fig. 6B), c-Src, and the degree of association between β1-integrin and c-Src was unchanged in cells expressing ΔP1/ΔP2 α1C-CaL (Supplemental Fig. S4B, quantified data not shown). These results suggest that the PRDs in the α1C-CaL COOH terminus are partially required for associations between α1C-CaL and β1-integrin and between α1C-CaL and c-Src.

Functional modulation of CaL current by α5β1-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 CaL current following α5β1-integrin engagement. As shown in Fig. 7A, the application of α5β1-integrin antibody to the bath solution potentiated whole cell CaL current in HEK293-T cells expressing WT-CaL, consistent with our previous findings (14). To our surprise, no significant electrophysiological difference was observed between WT-CaL and P1/P2-CaL (82% of WT) in the degree of current potentiation by integrin ligation, suggesting that the COOH-terminal PRDs are not required for potentiation of CaL current by integrin ligation. When the degree of CaL current potentiation following α5β1-integrin ligation in the mutant constructs was normalized to that of WT, both the truncated α1C-CaL and S1901A/Y2122F-CaL mutants displayed significant impairment in the amount of current potentiation by integrin ligation (Fig. 7B, 9% and 1% potentiation of WT-CaL, respectively; see also Ref. 14). The above results confirm that S1901 and Y2122 residues in the COOH terminus of α1C-CaL are required for Ca2+ current potentiation following α5β1-integrin engagement. However, selective deletion of the two PRDs in the COOH terminus of α1C-CaL does not impair the current potentiation by α5β1-integrin engagement.
Immunofluorescence imaging analysis demonstrates association of CaL with β1-integrin on the plasma membrane. The COOH terminus of α1C-CaL 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 α1C-CaL and its association with β1-integrin. Immunofluorescence confocal microscopy (IF) was performed to determine the expression, distribution, and relative localization of α1C-CaL and β1-integrin on the plasma membrane. Images of cells without staining or cells stained with either anti-mouse IgG, anti-rabbit IgG, or the combination membrane targeting of α1C-CaL, or truncated α1C-CaL, and ICA to determine the degree of α1C-CaL-β1-integrin colocalization, as listed in Table 1. The degrees of α1C-CaL colocalization with β1-integrin in WT-CaL, truncated α1C-CaL, and ΔP1/ΔP2-CaL, or S1901A/Y2122F-CaL, or ΔP1/ΔP2 α1C-CaL 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 α1C-CaL association with β1-integrin were observed in the truncated α1C-CaL, S1901A/Y2122F-CaL, truncated α1C-CaL, or ΔP1/ΔP2 α1C-CaL, compared with WT-CaL.

When we examined the association between WT α1C-CaL and endogenous β1-integrin on the plasma membrane, a moderate degree of colocalization was observed. To quantify the degree of colocalization between α1C-CaL and β1-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 α1C-CaL and β1-integrin. We used Pearson’s coefficient (R), Mander’s coefficient (R), and ICA to determine the degree of α1C-CaL-β1-integrin colocalization, as listed in Table 1. The degrees of α1C-CaL colocalization with β1-integrin in WT-CaL, truncated α1C-CaL, S1901A/Y2122F-CaL, or ΔP1/ΔP2 α1C-CaL were significantly less than the degree of colocalization between paxillin and vinculin (60–88% of paxillin-vinculin colocalization).

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phorylation of c-Src. Collectively, our data support the concept targeting of \( \alpha_1 \beta_1 \)-integrin. \( \alpha_1 \beta_1 \)-integrin 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 CaL.

We present evidence that the pore-forming \( \alpha_{1C} \)-subunit of the L-type calcium channel (\( \alpha_{1C} \)-CaL) associates with \( \beta_1 \)-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 CaL. The latter result provides evidence for a unique local functional regulation of CaL by \( \alpha_1 \beta_1 \)-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 CaL population, which is assembled upon integrin ligation and required for potentiation of CaL current following \( \alpha_1 \beta_1 \)-integrin engagement.

In this study, three different approaches, IP, IF, and patch-clamp recording of CaL current, were used to assess structural and functional interactions between \( \alpha_{1C} \)-CaL and \( \alpha_1 \beta_1 \)-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 \( \alpha_1 \beta_1 \)-integrin? What signaling components are required to mediate \( \alpha_{1C} \)-CaL association with \( \alpha_1 \beta_1 \)-integrin upon adhesion to FN? How does the spatial interaction between \( \alpha_{1C} \)-CaL and \( \alpha_1 \beta_1 \)-integrin contribute to the functional regulation of CaL? What is the physiological significance of \( \alpha_{1C} \)-CaL association with \( \alpha_1 \beta_1 \)-integrin? These issues will be addressed in the following sections.

**DISCUSSION**

We present evidence that the pore-forming \( \alpha_{1C} \)-subunit of the L-type calcium channel (\( \alpha_{1C} \)-CaL) associates with \( \beta_1 \)-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 CaL.

**Fig. 7.** Electrophysiological protocols to determine the effects of COOH terminus-specific regions on modulation of CaL current by \( \alpha_1 \beta_1 \)-integrin. A: representative recordings of whole cell CaL current from HEK293-T cells expressing WT-CaL, \( \Delta P1/\Delta P2 \)-CaL (left) or \( \Delta P1/\Delta P2 \)-CaL (right). Top traces in both panels show the amount of basal current in cells expressing WT- or \( \Delta P1/\Delta P2 \)-CaL, and bottom traces show the current potentiation by \( \alpha_1 \beta_1 \)-integrin Ab (10 \( \mu \)g/ml). No substantial difference in the relative amount of CaL current potentiated was observed in cells expressing \( \Delta P1/\Delta P2 \)-CaL, compared with WT-CaL. B: summary graph showing peak current potentiation by \( \alpha_1 \beta_1 \)-integrin in WT-CaL, truncated \( \alpha_{1C} \)-CaL, or \( \Delta P1/\Delta P2 \)-CaL. CaL current potentiated by \( \alpha_1 \beta_1 \)-integrin Ab was significantly attenuated in truncated \( \alpha_{1C} \)-CaL or in \( \alpha_1 \beta_1 \)-integrin Ab, but not in \( \Delta P1/\Delta P2 \)-CaL. Number of cells recorded is as follows: WT-CaL, \( n = 18 \); \( \Delta P1/\Delta P2 \)-CaL, \( n = 8 \) in cells expressing \( \Delta P1/\Delta P2 \)-CaL, \( S^901A/Y^{2122F} \)-CaL, or truncated \( \alpha_{1C} \)-CaL. *P < 0.05 vs. WT-CaL.
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⁺/H11001 channel (GIRK).

This RGD sequence mediates the direct interaction between GIRK and the /H92521-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 CaL isoforms, so that direct binding of α₁C-CaL to RGD-binding integrins is unlikely due to potential steric hindrance. Thus, an indirect association between α₁C-CaL and /H92521-integrin through cytoplasmic or cytoskeletal proteins seems more probable.

To determine regions in α₁C-CaL that provide binding motifs for other focal adhesion proteins that are known to associate with α₁β₁-integrin, we used a site-directed mutagenesis strategy. On the basis of differences in the amount of coimmuno-

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**Fig. 8.** Confocal immunofluorescence image analysis to determine the distribution and association of α₁C-CaL and β₁-integrin on the plasma membrane. Representative confocal immunofluorescence images from the cells expressing WT-, truncated α₁C-, S¹⁹⁰₁/A/Y²¹²²F-, or ΔP1/ΔP2-CaL, α₁C and endogenous β₁-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 α₁C-CaL with β₁-integrin on the membrane appears as yellow to orange punctate staining (c, g, k, and o). A noticeably higher degree of α₁C-CaL colocalization with β₁-integrin was seen in cells expressing WT-α₁C-CaL, compared with mutant α₁C-CaL 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 α₁C-CaL and β₁-integrin in the WT- or mutant α₁C-CaL-expressing cells. Merged image with nuclear counterstaining in blue appears in t. Magnification, ×63 oil; scale bar, 10 μm.
Table 1. Semiquantitative analysis of CaL association with β1-integrin

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<tr>
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<th>Paxillin-vinculin association</th>
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<th>ICA</th>
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<tr>
<td></td>
<td>Rr, % of Pax + Vin</td>
<td>ICA %</td>
<td>ICA, % of Pax + Vin</td>
<td>ICA %</td>
</tr>
<tr>
<td>Paxillin-vinculin</td>
<td>100</td>
<td>0.251 ± 0.021</td>
<td>100</td>
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<tr>
<td>WT</td>
<td>60.6 ± 2.7</td>
<td>0.22 ± 0.009</td>
<td>85.1 ± 4.2</td>
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<tr>
<td>Truncated-αIC</td>
<td>59.2 ± 4.8</td>
<td>0.211 ± 0.001</td>
<td>84.1 ± 5.0</td>
<td>84.1 ± 5.0</td>
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<tr>
<td>S1901A/Y2122F</td>
<td>56.5 ± 4.5</td>
<td>0.21 ± 0.011</td>
<td>84.8 ± 5.3</td>
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<td>ΔP1/ΔP2</td>
<td>55.4 ± 5.1</td>
<td>0.185 ± 0.013</td>
<td>75.2 ± 7.4</td>
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Values are means ± SE, taken from at least eight cells in either wild-type (WT) or mutant L-type voltage-gated Cav1.2 calcium channel (CaL) per experiment. The degree of CaL association with β1-integrin was normalized using the association of paxillin (Pax) and vinculin (Vinc) as a reference and set as 100% for Pearson’s, Mander’s, or intensity correlation analysis (ICA). The value of CaL association with β1-integrin in WT, truncated αIC-, S1901A/Y2122F, or ΔP1/ΔP2-CaL, is presented as the percentage ± SE of paxillin-vinculin association. Rr, Pearson’s coefficient; R, Mander’s coefficient. Statistical analysis was performed on the basis of the average from at least four individual experiments.

precipitated α1C-CaL with β1-integrin in WT versus mutated α1C-CaL. We identified several regions in the α1C-CaL COOH terminus that are involved in the interaction of α1C-CaL with αβ1-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 α1C-CaL with αβ1-integrin.

The roles of PKA in the functional regulation of CaL and its association with αβ1-integrin. PKA is known to regulate all three CaL isoforms. In neurons, PKA colocalizes with adenylate cyclase, β2-adrenergic receptor (β2-AR), A-kinase anchoring protein (AKAP), and protein phosphatase (PP2A) in a macromolecular signaling complex; PKA mediates the increase in neuronal CaL current following stimulation of β2-AR (6). Our results suggest that PKA is also a component of another macromolecular complex containing various focal adhesion proteins recruited following ligation of αβ1-integrin and that signaling between proteins in this complex also can exert a significant degree of control over CaL function. Evidence to support this conclusion derives from two aspects of our results. First, reduced association between α1C-CaL and β1-integrin was observed in an α1C-CaL mutant with altered PKA and c-Src phosphorylation sites. Second, our previous electrophysiological studies demonstrated a substantial reduction in the amount of CaL current that was potentiated following ligation of αβ1-integrin when α1C-CaL mutants with altered PKA and/or c-Src phosphorylation were used (14). When S1901 in α1C is mutated, it not only leads to reduction in the association between α1C-CaL and β1-integrin, but also to reduction in the phosphorylation of CaL by PKA (14). Findings from other laboratories also support the notion that S1901 in the COOH terminus of α1C-CaL is a focal assembly point for PKA, AKAP, PP2A, and WAVE/WASP, which collectively modulate CaL 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 β1-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 CaL function is influenced by αβ1-integrin signaling.

The roles of PRDs and c-Src in functional regulation of CaL and its association with αβ1-integrin. c-Src is another kinase that is implicated in the signaling pathway between αβ1-integrin and CaL. The involvement of c-Src is supported by several findings in the present study: 1) co-IP of α1C-CaL with c-Src in arteriolar SM appears to be independent of FN; 2) co-IP of α1C-CaL and phosphorylated c-Src is only observed in arteriolar SM incubated with FN; 3) co-IP of α1C-CaL 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 of α1C-CaL (Y2122) leads to reduced co-IP of α1C-CaL with β1-integrin and of α1C-CaL with c-Src. These results are consistent with previous findings that purified c-Src associates with the COOH terminus of α1C-CaL (19, 20) and that a basal level of CaL 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 CaL current following αβ1-integrin ligation is significantly reduced in Y2122F α1C-CaL constructs (14). Thus, preventing this tyrosine residue from being phosphorylated not only reduces the overall phosphorylation of CaL by exogenous c-Src, but also reduces the spatial interaction between α1C-CaL and β1-integrin and between α1C-CaL and endogenous c-Src, indicating that the interactions between α1C-CaL and β1-integrin and between α1C-CaL and
c-Src are required for current potentiation following αβ1-integrin ligation.

Our IP studies also identified a region in the α1c-CaL COOH terminus containing two PRDs (P1/P2) as another potential binding domain for c-Src. Reduced associations between α1c-CaL and β1-integrin and between α1c-CaL and c-Src were observed in the ΔP1/ΔP2 α1C-CaL mutant. Our results agree with a previous study demonstrating that fusion proteins containing α1c PRDs mediate interactions with c-Src in SY5Y cells expressing the neuronal CaL isofrom upon IGF stimulation (1). Taken together, the results suggest multiple roles for PRDs in association with the macromolecular complex containing β1-integrin, c-Src, and α1c-CaL.

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 α1c-CaL associates with FAK, our previous studies in vascular SMC revealed that a significant amount of the CaL current potentiation following αβ1-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 α1c-CaL via binding to PRDs (9). On the basis of the above, the PRDs in the α1c-CaL appear to play significant roles in the assembly of a macromolecular complex containing CaL, αβ1-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 α1c-CaL with αβ1-integrin may be more complicated than at first suggested by the preceding discussion. When the ΔP1/ΔP2 α1C-CaL mutant is expressed, the IP results (Fig. 6, A and C) reveal a reduced association between α1C-CaL and β1-integrin and between α1C-CaL and c-Src, compared with WT α1C-CaL, whereas the electrophysiological data (Fig. 7, A and B) reveal no substantial impairment in the degree of CaL current potentiation following αβ1-integrin ligation. The discrepancy may arise from two issues. First, the PRD regions that we deleted from α1C-CaL 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 CaL isofrom leads to enhancement of basal CaL 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 isofroms of the CaL 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 CaL 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 α1c-CaL (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 CaL, which consequently alters CaL function. Our IP results revealed that deletion of the COOH-terminal PRDs only reduced c-Src coassociation with α1C-CaL by 42%, even when the amount of truncated COOH-terminal α1C-CaL mutants for IP was increased by twofold (Supplemental Fig. S2, A and C). Thus, deletion of only the PRDs in the COOH terminus of α1C-CaL may not be sufficient to fully abolish the potentiation in CaL current following αβ1-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 α1C-CaL.

Reduced association between α1c-CaL and β1-integrin is not due to faulty membrane targeting of CaL. The amino acid residues 1623–1733 in the COOH terminus of cardiac α1C-CaL have been shown to mediate the membrane targeting of the channel (11). Using IF, we detected no significant difference in membrane targeting of α1C-CaL or β1-integrin in S1901A/Y2122F-CaL, truncated α1C-CaL at amino acid residue 1862, or ΔP1/ΔP2-CaL, compared with WT-CaL. Our results suggest that the reduced association between α1C-CaL and β1-integrin observed in the above mentioned α1C-CaL mutants is not due to unsuccessful membrane targeting of α1C-CaL. Our findings agree with those of Gao et al. (11) that the regions in the COOH terminus required for successful membrane targeting of cardiac CaL are amino acid residues 1623–1733. Because those specific regions are not altered in our mutant α1C-CaL constructs, the reduced association between α1C-CaL and β1-integrin in our mutant constructs are unlikely attributable to incorrect membrane targeting of CaL. In addition, our IP results also suggest that the association between α1C-CaL and β1-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 α1C-CaL. These results suggest that αβ1-integrin modulates CaL 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, αβ1-integrin, and CaL in the cytoplasm following αβ1-integrin engagement appears to play an important role in CaL current potentiation by αβ1-integrin.

Physiological significance of CaL association with integrin. Our findings of an association between CaL and β1-integrin and between CaL and c-Src provide evidence for a macromolecular signaling complex composed of αβ1-integrin, CaL, c-Src, PKA, and possibly other focal adhesion components. Formation of this complex appears to be important for regulation of CaL function following integrin-ECM interaction. Our IP results are consistent with previous findings that c-Src is involved in the increase of CaL 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 αβ1-integrin to produce additive or synergistic potentiation of CaL function by integrin.

In summary, this is the first study to provide evidence for the association of α1c-CaL with αβ1-integrin or α1C-CaL with c-Src in arteriolar smooth muscle. This association depends on...
engagement of α3β1-integrin by FN and specific regions in the α1C-Cα1.2 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 α3β1-integrin and that the interactions of proteins within this complex contribute to modulation of CaL function. Our study provides new insights into a CaL macromolecular regulatory complex that is required for functional regulation of the channel following α3β1-integrin engagement.

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DISCLOSURES

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

REFERENCES


