Integrin Receptor Activation Triggers Converging Regulation of Cav1.2 Calcium Channels by c-Src and Protein Kinase A Pathways*

Peichun Gui†, Xin Wu, Shizhang Ling‡, Stephanie C. Stotz§, Robert J. Winkfein†, Emily Wilson¶, George E. Davis‖, Andrew P. Braun‡‡, Gerald W. Zampolni¶¶ and Michael J. Davis††

From the †Department of Medical Pharmacology & Physiology, University of Missouri School of Medicine, Columbia, Missouri 65212, the ‡Department of Systems Biology & Translational Medicine, Texas A&M University College of Medicine, College Station, Texas 77840, and the §Smooth Muscle and Cellular and Molecular Neurobiology Research Groups, University of Calgary School of Medicine, Calgary, Alberta T2N 4N1, Canada

L-type, voltage-gated Ca2+ channels (CaL) play critical roles in brain and muscle cell excitability. Here we show that currents through heterologously expressed neuronal and smooth muscle CaL channel isoforms are acutely potentiated following α5β1 integrin activation. Only the α1c pore-forming channel subunit is critical for this process. Truncation and site-directed mutagenesis strategies reveal that regulation of Cav1.2 by α5β1 integrin requires phosphorylation of α1c C-terminal residues Ser1901 and Tyr2122. These sites are known to be phosphorylated by protein kinase A (PKA) and c-Src, respectively, and are conserved between rat neuronal (Cav1.2c) and smooth muscle (Cav1.2b) isoforms. Kinase assays are consistent with phosphorylation of these two residues by PKA and c-Src. Following α5β1 integrin activation, native CaL channels in rat arteriolar smooth muscle exhibit potentiation that is completely blocked by combined PKA and Src inhibition. Our results demonstrate that integrin-ECM interactions are a common mechanism for the acute regulation of CaL channels in brain and muscle. These findings are consistent with the growing recognition of the importance of integrin-channel interactions in cellular responses to injury and the acute control of synaptic and blood vessel function.

Voltage-gated calcium channels play critical roles in the regulation of calcium entry across the plasma membranes of excitable cells. L-type calcium channels (CaL) are heteromeric transmembrane proteins composed of a pore-forming α1C (Cav1.2) subunit along withaccessory β, α1, δ, and sometimes γ subunits (1, 2). The α1C subunit contains four highly conserved repeat regions with 24 membrane-spanning domains, in addition to a variable length N terminus and relatively long, intracellular C terminus. The three α1C isoforms (neuronal, Cav1.2c; smooth muscle, Cav1.2b; cardiac, Cav1.2a) exhibit significant sequence differences in their N and C termini but all are regulated by intracellular kinases in ways that uniquely determine calcium entry and cell excitability.

The regulation of CaL channels by serine-threonine kinases has been extensively investigated. PKG phosphorylates a conserved serine residue in the cytoplasmic I-1 linker (3) of all three α1c isoforms, leading to inhibition of current. PKC phosphorylates N-terminal threonine residues in cardiac and smooth muscle isoforms (4–6) leading in most cases to potentiation of current. PKA phosphorylates all three α1c isoforms at a conserved C-terminal serine (Ser1901 in Cav1.2c; Ser1928 in Cav1.2a), thereby mediating β-adrenergic potentiation of the calcium current in cardiac myocytes and neurons (7–9). PKA also regulates α1c in smooth muscle, but the functional consequences on calcium current are complicated by crossover activation of PKG, which is expressed at high levels in that tissue (10).

We recently demonstrated that CaL currents in vascular smooth muscle (VSM) are acutely regulated by the integrin class of cell adhesion molecules. CaL current is inhibited by ligands of αvβ3 integrin but potentiated by ligands of α5β1 and α4β1 integrins, including the abundant extracellular matrix (ECM) protein, fibronectin (11). α5β1-mediated potentiation depends critically on the integrin-associated tyrosine kinases c-Src and focal adhesion kinase (12). This mechanism is consistent with reports that α1c isoforms are potentiated by the growth factors, insulin-like growth factor-1 and platelet-derived growth factor (13–15), and that integrin-CaL channel interactions are required for neurotropin production (16) and acute control of synaptic function in the adult central nervous system (17–22).

Here, we present evidence that activation of α5β1 integrin acutely potentiates CaL channels in rat brain and smooth muscle. The effect can be reproduced fully in heterologously expressed neuronal and smooth muscle CaL isoforms and depends critically on phosphorylation of two specific C-terminal serine and tyrosine residues, indicating that integrin-ECM interactions are a common mechanism for acute regulation of CaL channels in brain and muscle.

EXPERIMENTAL PROCEDURES

Electrophysiology—Patch clamp recordings were made using EPC7 and EPC9 amplifiers controlled by pClamp (Axon Instruments) or Pulse (HEKA) software. Methods were mostly as described previously (12). Both perforated-patch and conventional whole cell modes were used. Pipettes were filled with Cs+ pipette solution (110 CsCl) containing (in
Regulation of Cav1.2 by α5β1 Integrin through PKA and c-Src

a. MS/nDB Neuron

b. VSM Cell

Heterologous Channel Expression—HEK-293 cells (tsA-201 line) were maintained at 30–37 °C in a 5% CO₂ incubator in supplemented Dulbecco’s modified Eagle’s medium, and transfected with Cav1.2 isoforms using CaPO₄ (for electrophysiology) or Lipofectamine 2000 (for Western blotting). Rat neuronal α₁C, β₁a, and α₂-δ DNA, subcloned into pCDNA3.1 vectors, were gifts from T. Snutch; rabbit SM α₁C, β₂a, and α₂-δ DNA were gifts from F. Hofmann and N. Klugbauer; human c-Src subcloned into Srα using the EcoRI site was a gift from D. Fujiia. Enhanced green fluorescent protein (EGFP) was used as a co-transfectant in all electrophysiological protocols to identify successfully transfected cells. Only single cells were used for electrophysiological protocols, typically 48–72 h after transfection.

Mutagenesis Methods—A PCR strategy was used to introduce stop codons at various positions in the Cav1.2 α₁C subunit C terminus. The wild type Cav1.2 clone contained a NotI site at the 3’ multicloning site. Thus, primer A was designed to anneal to the Cav1.2 antisense strand immediately upstream of the site intended for the stop codon. The primer included the stop codon and the sequence encoding a NotI restriction site. Primer B was designed to associate with the Cav1.2 residues upstream of both a unique SbfI restriction site and the placement of the intended stop codon. The resulting PCR product that extended from the SbfI site to the stop codon/NotI site was then sequenced, excised via SbfI and NotI, and subcloned into the original Cav1.2 construct via SbfI and NotI. Site-directed mutagenesis to eliminate phosphorylation consensus sites in the C terminus of Cav1.2 was conducted using the QuikChange site-directed mutagenesis kit (Stratagene), using the entire cDNA as a template. Subsequently, a fragment ranging from SbfI to NotI was sequenced to confirm the presence of the mutations, excised, and subcloned into the original WT Cav1.2 construct.

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assays—TSA-201 cells were lysed 48–72 h after transfection in Tris-buffered saline solution containing 1% Triton X-100 with a mixture of protease inhibitors (4 °C). Ca₂⁺ channels were immunoprecipitated by overnight

mM): 110 CsCl, 20 tetraethylammonium chloride, 10 EGTA, 2 MgCl₂, 10 HEPES, 1 CaCl₂ (pH 7.2 with CsOH). Perforated patch pipettes also contained 240 mg/ml amphotericin. Ba²⁺ was used as the charge carrier to increase the size of the inward current, and to minimize calcium-dependent inactivation of current. In experiments with VSM or HEK-293 cells, the bath solution (20 Ba²⁺) contained (in mM): 20 BaCl₂, 124 choline chloride, 10 HEPES, 15 d-glucose (pH 7.4 with triethanolamine-OH). In experiments with MS/nDB neurons, the bath solution (2 Ba²⁺) was similar except for containing 2 mM Ba²⁺.

Neuronal and VSM Cell Isolation—Basal forebrain neurons were isolated by microdissection of MS/nDB regions from coronal slices of rat (Sprague-Dawley) brain followed by incubation in trypsin and then trituration (23). Dissected segments of 1A and 2A arterioles from rat cremaster muscle were sequentially digested in low Ca²⁺ physiological saline solutions containing papain and then collagenase/ elastase. The resulting fragments were rinsed with low Ca²⁺ saline solution and gently triturated using a Pasteur pipette to release single, elongated VSM cells (12). All animal protocols conformed to the Public Health Service policy for the Humane Care and Use of Laboratory Animals and were approved by the respective university Animal Care Committees.

Reagents—The integrin ligands, a5 integrin monoclonal antibody (HM5-1, rat; BD Pharmingen) or α5β1 integrin polyclonal Ab (human, 10 µg/ml; Chemicon, Temecula, CA), were applied in soluble form or by biotin-streptavidin linking to polystyrene beads (3.2 µm, outer diameter) followed by addition of the cells (typically 5–10) to individual cells using gentle superfusion from a micropipette (11). Soluble reagents (PP2; Calbiochem) were applied using a picospritzer connected to micropipettes positioned close to the cell. In some protocols the solution in the recording pipette was exchanged with solutions containing inhibitors (PKA inhibitory peptide (PKI); Sigma) (Src SH2 inhibitory domain peptide; Calbiochem) over a 1–2 min period using a 2PK push-pull pipette exchange system (ALA Scientific Instruments, Westbury, NY).

Regulation of native neuronal and VSM cell Ca⁺⁺ currents by α5β1 integrin activation. a, whole cell Ba²⁺ current recordings from a rat MS/nDB neuron. α5β1 integrin was activated by applying beads coated with anti-α5 integrin Ab from a micropipette positioned close to the cell. Control trace was obtained 2 min after patch rupture; the α5-Ab trace was obtained 4 min after bead application. Current potentiation was 1.8-fold at test potential —10 mV. N-type and P/Q-type currents were blocked using 5 µM ω-conotoxin MVIIIC in the bath and Vh = —40 mV. The remaining current was blocked >95% by 1 µM nifedipine (not shown). The horizontal line indicates zero current level. The lower panel shows current-voltage relationships obtained from the same cell using a ramp protocol (—100 to +40 mV over 100 ms; Vh = —40 mV). Pipette, 110 Cs⁺; bath, 2 mM Ba²⁺, b, whole cell Ba²⁺ current recordings from a rat arteriolar smooth muscle cell before and after α5β1 integrin activation. Ba²⁺ current potentiation was 2.7-fold 4 min after application of α5β1 Ab beads. The lower panel shows current-voltage relationship from the same cell obtained using a ramp protocol (Vh = —80 mV). Pipette, 110 Cs⁺; bath, 20 mM Ba²⁺.
incubation with αIC Ab (Chemicon) followed by addition of protein A/G beads, pelleting, washing, and resuspension in Laemmli sample buffer. For immunoblotting, lysates or immunoprecipitates (IPs) were separated by SDS-PAGE using 6% acrylamide gels, transferred to nitrocellulose, and probed with appropriate primary and secondary Ab before application of SuperSignal chemiluminescent reagent and exposure to x-ray film. c-Src or cAK-induced phosphorylation was carried out at 30 °C in standard assay solution by incubation of IP complexes with 10 μM [γ-32P]ATP in the presence of purified recombinant human c-Src or cAK catalytic subunit purified from bovine heart, respectively (24). After separating the phosphorylation mixture by SDS-PAGE, the gels were exposed to x-ray film for 9–36 h at −20 °C; densitometry was performed using Scion software or, in some cases, a PhosphorImager.

RESULTS

Activation of α5β1 Integrin in Brain and VSM Potentiates CaL Current—Neurons (Fig. 1a) were acutely dissociated from the medial septum/diagonal band nucleus (MS/DBN) of the rat. CaL currents were measured using whole cell patch clamp techniques in the presence of 5 μM α-conotoxin MVIIIC and a holding potential of −40 mV to block N and P/Q channels (25). α5β1 integrins were activated by micropipette application of beads coated with anti-α5 integrin Ab (α5-Ab, a multivalent α5β1 ligand). Bead attachment to the neuron whose current is shown in Fig. 1a resulted in 1.6-fold potentiation of CaL current that peaked 3–5 min after bead application; potentiation averaged 1.43 ± 0.11-fold in 4 cells. I-V relationships of whole cells before (control) and after attachment of α5 beads are illustrated at the bottom of Fig. 1a. Potentiation did not appear to be associated with a significant shift in voltage sensitivity of the channel.

In rat VSM cells, the application of α5 integrin Ab on beads also resulted in time-dependent potentiation of the whole cell CaL current. Peak potentiation of the CaL current was 1.8-fold for the cell shown in Fig. 1b, whereas the average potentiation was 1.64 ± 0.05-fold in 5 cells. This effect was specific for integrin engagement because uncoated beads or beads coated with bovine serum albumin had no significant effect on current. In addition, a non-integrin binding Ab, in soluble form or coated onto beads, was without a significant effect on current (11).

Potentiation of Heterologously Expressed Cav1.2 by Integrin Activation—To explore the mechanism of current potentiation, CaL subunits were subsequently expressed in tsA-201 cells, which constitutively express α5β1 integrin and demonstrate α5β1 integrin-dependent adhesion (not shown). Transient transfection with the neuronal αIC subunit (Cav1.2c) alone resulted in relatively small currents that were potentiated 1.90 ± 0.13-fold by application of soluble α5β1 integrin Ab (human; Fig. 2a, left). Co-expression of β1A Ab alone or β1A Ab together with α5β1 Ab resulted in potentiation 1.85 ± 0.09-fold, respectively. Pipette, 110 CsCl; bath, 20 mM Ba2+.

Untransfected cells had no detectable voltage-gated Ba2+ current under the same conditions (data not shown). * Significant difference from basal current, p < 0.05.
alone or α1C-b, together with β2a and α2-δ1 subunits. The data are summarized in Fig. 3b, where the potentiation averaged 1.99 ± 0.20- and 1.94 ± 0.14-fold, respectively.

**Potentiation of Cav1.2 Requires the Distal C Terminus of the α1C Subunit**—Compared with smooth muscle and cardiac isoforms, the neuronal channel (Cav1.2c) has 27 fewer amino acid residues on the N terminus (28), including potential PKC phosphorylation sites (6, 29). The comparable potentiation of neuronal and smooth muscle isoforms argues that the N terminus is not the regulatory site for α5β1 integrin-mediated potentiation.

Subsequently, a series of successive, C-terminal truncation mutants of Cav1.2c were created and their responses to α5β1 integrin activation tested. As shown in Fig. 4, the truncation mutant Stop 5, which terminated proximal to the canonical PKA phosphorylation site (9) at Ser1901 (equivalent to Ser1928 on cardiac Cav1.2a) exhibited a loss of significant potentiation by α5β1 integrin Ab. In contrast, truncations distal to the PKA phosphorylation site (Stop 6–8), including those missing a proline-rich domain (30), resulted in a significantly reduced (≤50%), but not completely abolished, response to integrin activation, as compared with wild-type (WT) Cav1.2c. Each of the Stop 6–8 constructs was missing the putative tyrosine phosphorylation site at Tyr2122 previously identified as a regulatory site for insulin-like growth factor-1 (14). These results suggest that two different regulatory sites on the C terminus of Cav1.2 are required for full potentiation following α5β1 integrin activation, with one of the sites being Ser1901 (Cav1.2c). Consistent with this conclusion is the additional observation that inclusion of PKI in the patch pipette abolished the remaining potentiation associated with integrin activation in the Stop 6–8 constructs (Fig. 4c).

**Potentiation of Cav1.2c Depends Partially on α1C-c Tyr2122**—Our previous work suggested that potentiation of CaL in rat smooth muscle was partially but incompletely blocked by tyrosine kinase inhibitors (12). Because only two tyrosine residues in rat Cav1.2c are distal to Ser1901, we constructed single-site mutants in which each of those residues was substituted with phenylalanine (Y2122F-α1C and Y2139F-α1C). After expression, current through the Y2122F-α1C construct continued to be potentiated by the α5β1 integrin Ab, but potentiation was only 1.39 ± 0.06-fold above basal current, compared with 1.90 ± 0.13-fold for WT-α1C (Fig. 5, a and b). In contrast, the magnitude of potentiation of Y2139-α1C was statistically indistinguishable from that for WT-α1C (1.86 ± 0.09- versus 1.90 ± 0.13-fold, respectively). To further verify that Tyr2122 was critical for full potentiation by α5β1 integrin, we recorded current through WT-α1C, while dialyzing the cells with a decay peptide containing the same amino acid sequence as rat WT-α1C-c. This peptide was previously found to interfere with potentiation of native CaL channels in smooth muscle (12), and here it reduced integrin-induced potentiation of WT-α1C by ~50% (to 1.43 ± 0.05-fold above basal). As controls, neither an identical peptide containing a Y2122F substitution, nor a scrambled peptide, had any effect on integrin-induced potentiation of WT-α1C (Fig. 5b). Collectively, these results suggest that Tyr2122 is required for a significant fraction of, but not complete, potentiation of α1C-c following α5β1 integrin activation.

As an additional approach to testing whether the Tyr2122 residue of α1C-c was phosphorylated following integrin activation, we immunoprecipitated the channel after its expression in HEK-293 cells and performed in vitro phosphorylation assays with [γ-32P]ATP in the presence or absence of purified Src tyrosine kinase. Radiolabeled phosphate incorporation into Y2122F-α1C was reduced to 39 ± 6% of the level in WT-α1C, whereas [γ-32P]ATP incorporation into Y2139F-α1C averaged 120 ± 26% of that in WT-α1C (Fig. 5c, right). Phosphorylation of WT-α1C and Y2139F-α1C could also be detected (albeit at much lower levels) in the absence of exogenous c-Src (Fig. 5c, left), but it was nearly undetectable in Y2122F-α1C under comparable conditions. These results are consistent with the conclusion that phosphorylation of the Tyr2122 residue in α1C-c by c-Src (or other tyrosine kinases) is part of the mechanism for integrin-induced potentiation of the CaL current.

**Potentiation of Cav1.2c Is Partly Mediated by c-Src**—We further explored the role of c-Src in integrin-induced potentiation of CaL by co-expressing various c-Src constructs along with WT-α1C in HEK-293 cells (Fig. 6, a and b). Co-expression of WT-Src (human) with WT-α1C resulted in a significantly enhanced potentiation of current following integrin activation (2.34 ± 0.13-fold) as compared with the magnitude of potentiation when WT-α1C was expressed alone (1.90 ± 0.13-fold). In contrast, co-expression of a kinase-dead (Kd) form of c-Src (24), along with WT-α1C, resulted in ~50% reduction of potentiation of current after integrin activation (to 1.43 ± 0.05-fold). Although we attempted various additional methods of enhancing the amount of...
Regulation of Cav1.2 by α5β1 Integrin through PKA and c-Src

**a.**

- WT-α1C-c
- Stop 5
- Stop 6
- Stop 7
- Stop 8

**b.**

- WT-α1C-c
- Stop 5
- Stop 6
- Stop 7
- Stop 8

**c.**

- α1C subunit only
- α1C + β1b + α2b

**FIGURE 4.** Critical C-terminal domains required for integrin-induced potentiation of Cav1.2c. a, representative recordings from HEK-293 cells transfected with α1C-β1b and α5-8 subunits. Left-most trace shows the degree of Ba²⁺ current potentiation by soluble α5β1 integrin (10 μg/ml) in cells expressing WT-α1C-c, where peak potentiation averaged 1.90 ± 0.13-fold (n = 18). The other traces show examples of potentiation for various truncated α1C-c constructs as illustrated in b. The Stop 5 construct was WT α1C-c truncated at residue 1926, just proximal to the consensus PKA phosphorylation site (Ser1900); no significant potentiation was observed for Stop 5 in response to α5β1 integrin activation. The Stop 6 construct was truncated at residue 1929, just distal to the PKA site. The Stop 7 construct was truncated at residue 1999, just distal to two proline-rich domains (P1 and P2, spanning residues 1948–1973). The Stop 8 construct was truncated at residue 2066, just proximal to two tyrosine residues near the end of the C terminus. Potentiation was significantly reduced but not abolished in all Stop 6–8 constructs; however, PKI peptide pre-loaded into the recording pipette abolished the remaining potentiation in each case (e.g. 5th trace). The numbering schemes for both rat neuronal (α1C-c) and rat smooth muscle (α1C-c) isoforms are shown for reference (EF and DCT-i refer to an EF-hand motif and distal C terminus inhibitory domain, respectively). c, summary data for the constructs shown in comparison to basal current (prior to α5β1 Ab application) and potentiation of WT α1C-c (1–5 min after α5β1 Ab application). Left graph represents cells transfected with various α1C-c constructs plus wild type β1b and α5-8 subunits. Pipette, 110 Cs⁺; bath, 20 mV Ba²⁺; voltage step same as in upper left trace of panel a, *. significant difference from basal current, p < 0.05, #, significant difference from WT-α1C + α5β1 Ab, p < 0.05.

Kd-Src expression, we were unable to completely abolish the α5 integrin-induced potentiation of current (not shown). As an alternative approach to Kd-Src co-expression, we tested the effects of PP2 (100 nM), a membrane permeable inhibitor of Src family kinases, which reduced potentiation of WT-α1C-c by 40% following integrin activation (1.58 ± 0.06- versus 1.90 ± 0.13-fold). Collectively, these results are consistent with the conclusion that phosphorylation of the channel by c-Src accounts for some, but not all, of the potentiation following α5β1 integrin activation.

When the channel was immunoprecipitated and probed with an antiphosphotyrosine Ab, co-expression of c-Src with α1C significantly enhanced tyrosine phosphorylation of α1C (by ~5-fold; Fig. 6c). However, when Kd-Src was co-expressed instead, tyrosine phosphorylation of α1C was undetectable under comparable conditions. Although tyrosine phosphorylation of α1C by endogenous Src (Fig. 6c, lane 1) is not apparent in Fig. 6, longer film exposure times revealed detectable phosphorylation of the channel in the absence of c-Src co-transfection (not shown).

**Full Potentiation of Cav1.2c Depends on Dual Phosphorylation of α1C Residues Ser1900 and Tyr2122**—The results shown in Figs. 4 and 5 collectively suggest that full potentiation of α1C-c by α5β1 integrin activation requires phosphorylation of both α1C residues, Ser1900 and Tyr2122. To directly test this hypothesis, we constructed an α1C mutant in which the PKA site was altered to alanine (S1901A-α1C-c) and a double mutant in which both the PKA and Src phosphorylation sites were altered (S1901A/Y2122F-α1C-c). We initially checked the mutants to verify that regulation by PKA was impaired in response to the membrane-permeable cAMP analog, 8-Br-cAMP (Fig. 7, a and b, top). 8-Br-cAMP (1 mM) potentiated the WT-α1C current by 1.64 ± 0.12-fold and this effect was completely abolished in both S1901A-α1C-c and S1901A/Y2122F-α1C-c constructs. No significant potentiation by 8-Br-cAMP was observed in cells transfected with WT-α1C if PKI was included in the recording pipette.

We also tested whether the S1901A-α1C-c mutant could be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (cAK). Compared with phosphorylation of WT-α1C-c, phosho-
Regulation of Cav1.2 by α5β1 Integrin through PKA and c-Src

FIGURE 5. Integrin-induced potentiation of Cav1.2 partly depends on phosphorylation of a C-terminal tyrosine residue. a, representative traces showing potentiation of Ba2+ current following α5β1 integrin activation in cells transfected with β1ab, α5β1 subunits plus WT-α1C, or single site α1C-c mutants containing Y2122F or Y2139F substitutions. Potentiation was reduced by ∼50% in Y2122F-α1C, but not significantly in Y2139F-α1C. Potentiation was also reduced in cells transfected with WT-α1C, when an 11-amino acid decoy peptide (DEDESCYVALGR; trace 4), but not a control peptide (DESCYVALGR; trace 5), was included in the recording pipette (50 μM for all). Pipette, 110 Cs+; bath, 20 Ba2+. b, summary data for peak current potentiation by α5β1 integrin Ab in the various α1C-c constructs described in panel a, plus an additional control in which the peptide sequence was scrambled (LVCGAEYDNGS). c, in vitro phosphorylation of expressed WT or mutant α1C channels containing Tyr2122F mutations. Following co-expression in HEK cells of α1C, β1ab, and α5β1 subunits, the cells were lysed and the channels were IP using an α1C Ab and incubated with [γ-32P]ATP (upper bands) at 37 °C for 30 min in the presence or absence of purified pp60c-Src. Proteins were separated using SDS-PAGE and transferred to nitrocellulose. Lower bands (Blot: α1C) show the amount of immunoprecipitated α1C subunit detected in each sample after stripping and reprobing with α1C Ab. Low levels of [32P]ATP incorporation into the channel were detectable in the absence of c-Src (left lanes) but the signal increased dramatically in the presence of c-Src (right lanes). Relative to control (WT-α1C + Src lane), phosphorylation of the Y2122F-α1C and Y2139F-α1C mutant channels was 39 ± 6 and 120 ± 26%, respectively (n = 3). In the two lanes denoted WT-α1C glu-glu, IPs from WT-α1C-transfected cells were performed using a control Ab versus an irrelevant Glu-Glu epitope tag. Although this Ab did not IP any detectable α1C subunit, a nonspecific, 200-kDa phosphoprotein was observed in the presence of [γ-32P]ATP + c-Src Pipette; 110 Cs+; bath, 20 mM Ba2+; voltage step same as in upper left trace of panel a. *, significant difference from basal current, p < 0.05; #, significant difference from WT-α1C + α5β1 Ab, p < 0.05.

Finally, we examined the effects of α5β1 integrin activation on whole cell current recorded from expressing channels mutated at the PKA regulatory site. Potentiation of the S1901A-α1C construct following integrin activation was reduced by ∼45% (1.90 ± 0.13- versus 1.47 ± 0.13-fold) compared with the potentiation of WT-α1C; however, currents were still significantly higher than basal. In contrast, in cells expressing S1901A/Y2122F-α1C, integrin activation did not result in any significant potentiation of current above basal levels (Fig. 7b, bottom, and c). To further test this idea, we tested five different methods of interfering with phosphorylation of α1C at Ser1901 and Tyr1212: S1901A-α1C plus PP2 added to the bath; S1901A-α1C plus co-expression of KD-Src; WT-α1C co-expressed with KD-Src plus PKI in the recording pipette; Y2122F-α1C plus PKI in the recording pipette; and the S1901A/Y2122F-α1C double mutant. In all five cases, no significant potentiation of current was observed following integrin activation (Fig. 7c). These results strongly support the conclusion that potentiation of the Cav1.2c current by α5β1 integrin activation requires dual phosphorylation of sites Ser1901 and Tyr1212 on α1C.

Potentiation of Native Ca2+ Channels Requires Both PKA and c-Src—The evidence for dual phosphorylation of α1C-c by PKA and c-Src underlying integrin-dependent potentiation of the Ca2+ current prompted a more careful examination of the magnitude and time course of native Ca2+ current potentiation in rat cells during inhibition of Src and PKA. Fig. 8a shows the time course of current potentiation following application of beads coated with α5β1 integrin Ab onto single vascular myocytes. Consistent with previous findings, potentiation peaked 3–4 min...
after bead application and then spontaneously declined (11) toward control levels. When Src was inhibited just before peak potentiation by PP2 (100 nM in the bath) or by a Src SH2 inhibitory peptide (2.7 μM in the recording pipette), 75 and 60% less current potentiation was observed, respectively, compared with that recorded in the absence of inhibitors (Fig. 8b). PKI alone, applied by exchange of the recording pipette solution, resulted in only 17% reduction of integrin-potentiated current. PP3 (100 nM), the inactive analog of PP2, was without significant effect (not shown) on either basal or integrin-potentiated current (12). However, the combination of PP2 and PKI peptide completely blocked integrin-induced potentiation of current (Fig. 8b). These results are consistent with those from heterologously expressed channels (Figs. 4–7) and suggest that the same dual-phosphorylation mechanisms are operating to regulate native CaL channels.

**DISCUSSION**

Our results demonstrate that neuronal and smooth muscle CaL channels are regulated by α5β1 integrin through an intracellular signaling pathway involving phosphorylation of the α1C channel subunit by PKA and c-Src. Integrin activation produces up to 2-fold potentiation of Ca2+ current over a relatively short time frame (1–5 min). Given the central role of CaL channels in nerve and smooth muscle excitability, the ECM-integrin-CaL signaling pathway is likely to have major effects on both short- and long-term control of [Ca2+]i, and Ca2+-dependent processes in these respective cell types.

**Ion Channel Regulation by Integrins**—A growing body of literature supports our conclusion that ion channels are acutely regulated by integrin-ECM interactions (31). β1 integrins are known to modulate excitatory synaptic transmission through an as yet defined role of c-Src (16, 22, 32), observations that are consistent with the well known regulation of ligand-gated channels by tyrosine kinases (33). Recently, β1 integrins have also been implicated in the regulation of several different K+ channels, with evidence for both physical (34, 35) and functional (36–38) associations between the channels and integrins.

Several independent lines of evidence support the idea that integrins also regulate CaL channels. For example, bidirectional regulation of native rat smooth muscle CaL channels is observed following engagement of α5β1, α4β1, and αvβ3 integrins with ECM proteins or integrin antibodies (11, 39). In the case of α5β1 integrin, selective activation by multivalent integrin ligands results in ~70% potentiation of CaL current...
The specific response to KCl, and leads to an increase in neuronal excitability (40). Integrin-mediated potentiation of Cav1.2 is prevented by blocking channel phosphorylation at two C-terminal sites. About 50% potentiation by 8-Br-cAMP (1 mM) from a picospritzer pipette produced 1.64 ± 0.12-fold potentiation of WT-α1C, expressed with β1S and α2δ-1 subunits. Potentiation was completely blocked in the S1901Aα1C mutant (second trace, top) and in the S1901A/Y2122Fα1C, double mutant (third trace, top). Potentiation was prevented in WT-α1C, with PKI peptide (2.7 μM) in the recording pipette. c, summary data showing effects of S1901A and S1901A/Y2122F mutations on current potentiation in response to α5β1 integrin activation. About 50% potentiation by α5β1 Ab remained in S1901Aα1C (panel b, bottom left trace); significantly less than in WT-α1C, but significantly greater than basal current. PKI peptide also blocked ~50% α5β1 Ab-mediated potentiation of WT-α1C current. In contrast, five different methods of blocking phosphorylation of both Ser1901 and Tyr2122 sites, including the S1901A/Y2122Fα1C, double mutant (panel b, bottom right trace) resulted in no significant current potentiation following α5β1 integrin activation. d, in vitro kinase assays comparing phosphorylation of immunoprecipitated WT and mutant channels by the purified catalytic subunit of cAK in the presence of [γ-32P]ATP. Phosphorylation of neither mutant (Y2122F or Y2139F) was significantly different than for WT-α1C. Glu-Glu Ab did not immunoprecipitate the channel and served as a control. Pipette, 110 Cs+; bath, 20 mM Ba2++; voltage step same as in upper left trace of panel b. *, significant difference from basal current, p < 0.05; #, significant difference from WT-α1C + 8-Br-cAMP (panel a) or from WT-α1C + α5β1 Ab (panel b), p < 0.05.

The specific integrins responsible have not yet been identified but cRGD peptide, which interacts with multiple integrins, produces dose-dependent, biphasic changes in HVA current (composed of both N- and L-type channels), suggesting that the responses are mediated by multiple integrins and/or multiple Ca2+ channel types. The regulation of mammalian cardiac Cav1.2 channels by muscarinic and adrenergic receptors is substantially influenced by the adhesion of cardiac myocytes to their substratum. Specifically, Ca2+ responses to β2 adrenergic agonists are potentiated in cells adhered to laminin or β1 integrin Ab (compared with adhesion on glass), whereas responses to acetylcholine are attenuated (41, 42). However, another study suggests that adhesion-dependent potentiation of cardiac Cav1.2 is not specific to integrins (43). In cardiomyocytes derived from embryonic stem cells, muscarinic inhibition of Cav1.2 current is absent in β1-integrin−/− cells (44), although the effect is at least partly due to defective coupling of Gαq to subsequent to β1 integrin knockout. In this context it is interesting that Gαq co-localizes extensively with the focal adhesion proteins talin and vinculin (45) known to be critically involved in integrin-mediated signaling (46). In E63 skeletal muscle cells, engagement of α7β1 integrin by laminin or α7-integrin Ab triggers both Ca2+ release and influx (27). Interestingly, the extracellular domain of α7β1 integrin associates with the dihydropyridine receptor/channel (α1C), which shares substantial sequence homology with Cav1.2. Collectively, these findings support our contention that the pathways elucidated in the present study may be common mechanisms for regulation of Ca2+ entry in many nerve and muscle cells.
Regulation of Cav1.2 by α5β1 Integrin through PKA and c-Src

Collectively, this evidence points to the possibility that Ca₉ channels co-localize with integrins and/or focal adhesion proteins in excitable cells and that Ca₉-integrin-focal adhesion signaling complexes potentially overlap with Ca₉-PKA-AKAP complexes. The activation of PKA and subsequent phosphorylation of Cav1.2 channels following integrin activation observed in our study are consistent with activation of the cAMP signaling cascade in endothelial cells following application of mechanical stress through RGD-containing integrins (63); indeed, the activation of the cAMP cascade required both integrin receptor occupation and intact Grx signaling. Thus, it is possible that scaffolding proteins such as WAVE1, which associate with both PKA and integrin-linked tyrosine kinases (64), will be found to play critical roles in the regulation of Ca₉ channels. The possible crossover between these signaling pathways will be an important issue to resolve with respect to the regulation of Ca₉ in excitable cells.

It is interesting that the heterologously expressed rat neuronal isoform (Cav1.2c) showed a similar degree of potentiation as native rat neuronal and VSM Ca₉ channels following α5β1 integrin activation (compare Fig. 1 with Figs. 2 and 3). Similar physiological responses might be predicted based on the fact that there is >99% sequence homology between the rat neuronal (Cav1.2c) and rat smooth muscle (Cav1.2b) isoforms. For other species, there is less (~80%) homology in the regulatory portion (distal 2/3) of the C terminus. Notably, rabbit Cav1.2b lacks the critical tyrosine residue corresponding to Tyr²¹²⁰ on Cav1.2c that mediates ~50% of integrin-induced potentiation and that has also been implicated in regulation of rat Cav1.2c by the growth factor IGF-1 (14). However, rabbit Cav1.2b is also potentiated to a comparable degree by α5β1 integrin activation (Fig. 3). Whether potentiation of rabbit Cav1.2b depends both on phosphorylation by PKA at Ser¹⁹⁹⁸ and c-Src at an alternate tyrosine residue is an important question that needs to be answered. However, it is also known that rabbit smooth muscle Ca₉ channels may be regulated by c-Src through more indirect mechanisms (4, 13, 65).

ECM-Integrin-Cav1.2 Signaling Axis in Cellular Responses to Injury—The regulation of Ca₉, channels by integrin-ECM interactions can potentially play a role in a number of important physiological processes in both the central nervous system and the cardiovascular system. In the brain, integrins are concentrated at sites of synaptic contact (66) and are critical for the formation, maturation, and maintenance of synaptic structure (67). For example, laminin, along with its receptor, α7β1 integrin, plays a role in the clustering of acetylcholine receptors at the neuromuscular junction (68). Integrin engagement is required under some conditions for neurotrophic signaling (16) and integrin clustering can initiate receptor tyrosine kinase signaling in the absence of neurotrophins, as has been documented for other growth factors in other cell systems (69, 70). In this context, integrin-Ca₉ channel interactions

FIGURE 8. Effects of c-Src and PKA inhibition on potentiation of native smooth muscle Ca₉ currents following α5β1 integrin activation. a, time course of Ba²⁺ current potentiation in unstimulated cells (open circles) and in response to beads coated with α5 integrin Ab added 4 min after patch rupture (open squares; ~80% potentiation of current is evident at 7–8 min). When α5-bead application was followed by introduction into the patch pipette of a peptide (10 μM) that selectively inhibits Src binding through its SH2 domain, beginning at 6 min, there was a significant reversal of potentiation (closed squares). b, summary of data from protocols as shown in panel a.currents are normalized to unstimulated basal current (i.e. open circles in b) or to the degree of current potentiation by α5-beads alone (i.e. open squares in a). Src SH2 peptide alone (in pipette) inhibited ~60% of potentiation following integrin activation by α5-beads on beads; PP2 (in bath) inhibition ~75% of potentiation; PKI peptide (in pipette) inhibited potentiation by 17%. In contrast, no considerable potentiation remained when the combination of PKI peptide (in pipette) and PP2 (in bath) was used. All recordings were made in the conventional whole cell mode to allow peptide access and therefore exhibited current run-up and run-down. Pipette, 110 Cs⁻; bath, 20 mM Ba²⁺; V₉ = −80 mV; test potential = +30 mV. * significant difference from control; p < 0.05.
appear to be critical for the up-regulation of brain-derived neurotrophic factor mRNA in hippocampal neurons (16). Finally, a number of studies have now confirmed that integrins are instrumental in modulating long term potentiation and therefore in controlling synaptic function in the adult central nervous system (17–22). However, whether their role involves changes in cellular adhesion or modulation of intracellular signaling has not yet been defined.

Integrin-Ca\textsubscript{\text{\text{2}}} channel interactions could also play a major role in the responses of neurons and blood vessels to injury and repair. Both neuronal activity and vascular reactivity are modulated by integrin signaling through the generation or exposure of new integrin ligands from limited degradation of extracellular matrix and/or turnover of new integrins (71–73). For example, successful axonal regeneration is highly correlated with the induction of integrins on the surface of peripheral neurons; therefore, peptides derived from ECM proteins have the potential to act as therapeutic agents for neuronal regeneration (74). In blood vessels, it is well established that VSM sensitivity to agonists and to act as therapeutic agents for neuronal regeneration (74).

**REFERENCES**

1. Koh, W. J., Elizion, P. T., and Schwartz, A. (1990) J. Biol. Chem. 265, 17786–17791
2. Catterall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
3. Jiang, L. H., Gawler, D. J., Hodson, N., Milligan, C. J., Pearson, H. A., Porter, V., and Wray, D. (2000) J. Biol. Chem. 275, 6135–6143
4. Keef, K. D., Hume, J. R., and Zhong, J. (2001) Annu. Rev. Physiol. 2, 353–364
5. Kamp, T. J., and Hell, J. W. (2000) Circ. Res. 86, 403–411
6. Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) J. Biol. Chem. 273, 17901–17909
7. Gao, T., Yatani, A., Dell’Acqua, M. L., Sako, H., Green, S. A., Dascal, N., Scott, J. D., and Hosey, M. (1997) Neuron 19, 185–196
8. Sculthorpeau, A., Scheuer, T., and Catterall, W. A. (1993) Nature 364, 240–243
9. Mitterdorfer, J., Froschmayr, M., Grabner, M., Moebius, F. F., Glossmann, H., and Streissler, J. (1998) Biochemistry 35, 9400–9406
10. Ruiz-Velasco, V., Zhong, J. M., Hume, J. R., and Keef, K. D. (1998) Circ. Res. 83, 557–565
11. Wu, X., Mofgod, J. E., Platts, S. H., Davis, M. E., Meinerger, A. G., and Davis, M. J. (1998) J. Biol. Chem. 273, 20328–20329
12. Hu, X.-Q., Singh, N., Mukhopadhyay, D., and Akbarali, H. I. (1998) J. Biol. Chem. 273, 5337–5342
13. Bente-Hanulec, K. K., Marshall, J., and Blair, L. A. C. (2000) J. Biol. Chem. 275, 20189–20196
14. Kramar, E. A., Bernard, J. A., Gall, C. M., and Lynch, G. (2002) J. Neurosci. 22, 30–39
Regulation of Cav1.2 by α5β1 Integrin through PKA and c-Src

68. Burkin, D. J., Gu, M., Hodges, B. L., Campanelli, J. T., and Kaufman, S. J. (1998) J. Cell Biol. 143, 1067–1075
69. Howe, A., Aplin, A. E., Alahari, S. K., and Juliano, R. L. (1998) Curr. Opin. Cell Biol. 10, 220–231
70. Juliano, R. L., and Haskill, S. (1993) J. Cell Biol. 120, 577–585
71. Davis, G. E., Bayless, K. J., Davis, M. J., and Meininger, G. A. (2000) Am. J. Pathol. 156, 1489–1498
72. Sage, H. (1997) Trends Cell Biol. 7, 182–186
73. Gualandris, A., Jones, T. E., Strickland, S., and Tsirka, S. E. (1996) J. Neurosci. 16, 2220–2225
74. Meiners, S., and Mercado, M. L. (2003) Mol. Neurobiol. 27, 177–196
75. Martinez-Lemus, L. A., Wu, X., Wilson, E., Hill, M. A., Davis, G. E., Davis, M. J., and Meininger, G. A. (2003) J. Vasc. Res. 40, 211–233
76. Intengan, H. D., and Schiffrin, E. L. (2000) Hypertension 36, 312–318