Voltage-gated Mobility of the Ca\textsuperscript{2+} Channel Cytoplasmic Tails and Its Regulatory Role*

Evgeny Kobrinsky, Elena Schwartz, Darrell R. Abernethy, and Nikolai M. Soldatov

From the National Institute on Aging, National Institutes of Health, Maryland 21224

The ion conductance of voltage-dependent ion channels is tightly regulated by the gating mechanism encoded in membrane voltage sensors and cytoplasmic gates of the pore-forming \( \alpha \) subunit (1). This voltage gating is due to the displacement of membrane charges (2) that has been documented as gating current (2) and further characterized as conformational rearrangements detected by spectroscopy of the attached fluorescent reporter molecules (3–6). However, little is known about functional role for state-dependent mobility of the channel cytoplasmic tails (7). Such motion of the L-type (Cav1.2) Ca\textsuperscript{2+} channel C-terminal tail that binds calmodulin (CaM)\textsuperscript{1} may serve to transfer a regulatory function to voltage-gated calcium channels associated with their gating is important for understanding of the molecular correlates of channel regulation and mechanisms of Ca\textsuperscript{2+} signaling. Spectral properties of the enhanced cyan (ECFP) and yellow fluorescent proteins (EYFP) (10) are well suited for measurements of molecular rearrangements by FRET (11). Here we genetically fused the cytoplasmic N and C termini of the human Ca\textsubscript{1.2} channel \( \alpha \)C pore-forming subunits with EYFP and ECFP, respectively. The labeled channels were then functionally expressed in COS1 cells. The rearrangements of the tails due to transition into the distinct functional states of the channel were monitored using FRET (12) in living cells under voltage clamp conditions. A regulatory role for the voltage-gated mobility of the C-terminal tail was then characterized.

EXPERIMENTAL PROCEDURES

Molecular Biology—(EYFP)\textsubscript{N-77pcDNA3} and (EYFP)\textsubscript{N-1C,IS-IV} expression plasmids were prepared in pcDNA3 vector essentially as described earlier (13, 14) using pEYFP vector (Clontech). To prepare the PH-EYFP and PH-ECFP expression plasmids, the 518-bp EcoRI/BamHI fragment of pPLCPH-enhanced green fluorescent protein (15) was ligated into the pECFP-1 and pEYFP-1 plasmids, respectively, as EcoRI/BamHI sites. To prepare the \( \alpha \)C\textsubscript{77}-ECFP\textsubscript{C} expression plasmid, the 1029-bp AatII/NotI fragment of 77pcDNA3 (13) was replaced by the pECFP vector amplified by PCR using 5’TCTCAAGGCCTAC-3’ was ligated into the 6115-bp MfeI/NotI fragment of 77pcDNA3 into the NotI and BgrI-cut PH-EYFP plasmid. To prepare (PH-EYFP)\textsubscript{N-1C,77}-ECFP\textsubscript{C} expression plasmid, the 4347-bp NotI/PpuMI fragment of 77CfpDNA3 was replaced into the respective sites of (PH-EYFP)\textsubscript{N-1C,77} expression plasmid, by 921-bp terminal AatII/XhoI PCR fragment with a unique NotI linker was ligated with the XhoI/NotI fragment of open reading frame of the PH-ECFP plasmid into the AatII NotI-cut pHLCC77 plasmid. Its 5096-nucleotide PsuMI/NotI fragment was then subcloned into 77pcDNA3 or (EYFP)\textsubscript{N-1C,77}-pcDNA3 to give the \( \alpha \)C\textsubscript{77}-PH-ECFP\textsubscript{C} and (EYPFP)\textsubscript{N-1C,77}-PH-ECFP\textsubscript{C} expression plasmids, respectively.

Electrophysiology—COS1 cells were grown on poly-\( \gamma \)-lysine-coated coverslips (MatTek) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected with pcDNA3 vectors coding for \( \alpha \)C, \( \beta _1 \) (17) (or \( \beta _2 \) or \( \beta _3 \), respectively), and \( \delta \) (18) subunits using the Effectene kit (Qiagen). Effectene was used for transfection to minimize autofluorescence added by many other transfection reagents. EGF receptor was co-expressed in tail-anchoring FRET experiments. To release pleckstrin homology (PH) domain, serum-deprived (24 h) cells were exposed to EGF (100 ng/ml). Ion currents were recorded using the Axopatch 200B amplifier (Axon Instruments) at 20–22°C by the whole-cell patch clamp method 48–72 h after transfection of COS1 cells. Voltage protocols were generated, and data were digitized, recorded, and analyzed using pClamp 8.1 software (Axon). The extracellular bath solution contained (in mM) NaCl, 100; BaCl\(_2\), 20; MgCl\(_2\), 1; glucose, 10; tetraethylammonium, 5; CaCl\(_2\), 1; 1,2-bis(\( \alpha \)-aminoethoxyvinyl)ethane-N,N,N’,N’-tetraacetate, 10; tetraethylammonium, 5; 1,2-bis(\( \alpha \)-aminoethoxyvinyl)ethane-N,N,N’,N’-tetraacetate, 10; tetraethylammonium, 5; CaCl\(_2\), 1. The electrodes had resistance 3–6 M\( \Omega \)s and were filled with pipette solution containing (in mM) CsCl, 110; MgATP, 5; 1,2-bis(\( \alpha \)-aminoethoxyvinyl)ethane-N,N,N’,N’-tetraacetate, 10; tetraethylammonium, 5; 1,2-bis(\( \alpha \)-aminoethoxyvinyl)ethane-N,N,N’,N’-tetraacetate, 10; tetraethylammonium, 5; CaCl\(_2\), 1. Currents were sampled at 2.5–5 kHz and filtered at 1 kHz.

FRET Imaging—Images were recorded with the Hamamatsu digital camera C4742-95 mounted on a Nikon epifluorescence microscope TE2000 equipped with multiple filter sets (Chroma Technology). C- Imaging (Compix) and MetaMorph (Universal Imaging) software were used to obtain and analyze images. The photobleaching experiments were conducted with the 100-watt mercury lamp. For all other experiments, a 75-watt xenon lamp was used. FRET was quantitated with three filter sets as follows: for EYFP cube, excitation filter 500/20 nm, dichroic beam splitter 515 nm, and emission filter 535/30 nm; for ECFP (ECFP/EYFP) cube, excitation 436/20 nm, dichroic beam splitter 455 nm, and emission filter 480/30 nm; for ECFP cube, excitation filter 458/20 nm, dichroic beam splitter 455 nm, and emission filter 480/40 nm. Regions of interest were selected using the C- Imaging software where intensity (I) from three filter sets was

\( \text{I} = a \text{I}_{\text{ECFP}} + b \text{I}_{\text{EYFP}} + c \text{I}_{\text{PH-EYFP}} \)

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† To whom correspondence should be addressed: NIA, National Institutes of Health, 5600 Nathan Shock Dr., Baltimore, MD 21224. Tel.: 410-558-8343; Fax: 410-558-8318; E-mail: soldatov@nrgc.nia.nih.gov.

‡ The abbreviations used are: CaM, calmodulin; FRET, fluorescence resonance energy transfer; CREB, CAMP-responsive element-binding protein; ECFP, enhanced cyan fluorescent proteins; EYFP, enhanced yellow fluorescent protein; nt, nucleotide; PH, pleckstrin homology; EGF, epidermal growth factor; ACh, acetylcholine; M1AChR, muscarinic acetylcholine receptor.

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determined after background subtraction. Corrected intensity of FRET ($I_{\text{FRET}}$) was calculated (20) as $I_{\text{FRET}} = I_{\text{ECFP}} \times 58.5\% - I_{\text{EYFP}} \times 11.5\%$). In the patch clamp experiments, the acquisitions of fluorescence ranging from 50 to 300 ms were obtained with simultaneous recording of the current at the indicated conditioning voltages under steady state. In some cases, the images were adjusted pixel-by-pixel using the reference channel of regions of interest. With the acceptor photobleaching, we used an excitation filter 436/10 nm and an emission filter 470/30 nm for ECFP and an excitation filter 500/10 nm and an emission filter 535/20 nm for EYFP. The apparent efficiency of FRET was calculated as $I_{\text{EYFP}}/\sqrt{I_{\text{ECFP}} \times I_{\text{EYFP}}}$. Where, $I_{\text{ECFP}}$ and $I_{\text{EYFP}}$ are intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. Normalization of the corrected FRET intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching, respectively. 

## RESULTS

### Acceptor Photobleaching Assay—Confirming earlier findings (13, 27), fusion of the green fluorescent protein variants to the cytoplasmic N and/or C termini did not compromise channel function. The electrophysiological properties of the (EYFP)$_{N}$–$\alpha_{1C,77}$(ECFP)$_{C}$ channel were similar to those of the wild-type channel (13) except for the acceleration by $\sim$15% of the Ba$^{2+}$ current inactivation (Table II). A direct FRET acceptor photobleaching assay provided strong evidence of FRET in the (EYFP)$_{N}$–$\alpha_{1C,77}$(ECFP)$_{C}$ channel (Fig. 1). Illumination of the transfected COS1 cells for 15 min with a mercury lamp caused on average $>90\%$ photobleaching of EYFP. The resulting increase in donor fluorescence (shown as yellow-green and yellow-red signals, Fig. 1A, c and e, respectively) is a direct demonstration of FRET (11). As a positive control, under the same conditions, FRET was observed (Fig. 1A, d and f) with the co-expressed mixture of the EYFP-PH and ECFP-PH domains of phospholipase Cβ1 (28). In both cases, FRET was confined to the plasma membrane region where the functional channel molecules reside. We found that the mixture of co-expressed (1:1) single N- and C-tail-labeled channel isoforms did not show substantial intermolecular FRET (Fig. 1B) indicating that with the double-labeled $\alpha_{1C}$ channels we recorded predominantly intramolecular FRET.

### Voltage-dependent FRET in the Inactivated State—FRET is the result of long range dipole-dipole interactions between fluorophores. FRET depends on the distance ($r^6$) between the donor and acceptor fluorophore and, to a lesser extent, the relative orientation ($r^2$) of the dipoles (12). To determine the differences in relative proximity and/or angular orientation of the tagged $\alpha_{1C}$ tails in the functionally distinct resting, conducting, and inactivated states of the channel, we combined FRET imaging with voltage clamp. We used quantitative analysis of reversibly changing FRET in place of acceptor photobleaching as the fluorophores became irreversibly damaged by photobleaching. Simultaneous monitoring of the Ba$^{2+}$ current provided a direct assessment of the Ca$^{2+}$ current transition into the resting, conducting, or inactivated state prior to FRET imaging. The images were acquired with a three-filter set system, successfully used elsewhere (20, 29), for sensitized acceptor emission. The recombinant channel, composed of the wild-type $\alpha_{1C,77}$ and accessory $\beta_{1}$ and $\alpha_{2\delta}$ subunits, was stabilized in either the resting or inactivated state by whole-cell voltage clamp at $-30$ or $+40$ mV, respectively. Depolarization to $+40$ mV was selected to evoke maximum activation of the channels under conditions when the state of inactivation could be monitored. Thus, the amplitudes of the Ba$^{2+}$ currents (Fig. 2d) were less than 50% of the maximum ones. The analysis of FRET images of a cell expressing the (EYFP)$_{N}$–$\alpha_{1C,77}$(ECFP)$_{C}$ chan-

| Corrected FRET | $a_{1C,77}/\beta_{1}$ | $a_{1C,77}/\beta_{2}$ | $a_{1C,76}/\beta_{1}$ |
|----------------|---------------------|---------------------|---------------------|
| $I_{\text{FRET}}$, $-40$ mV | $2.9 \pm 0.7$ (24) | $3.8 \pm 0.8$ (15) | $7.4 \pm 0.9$ (19) |
| $I_{\text{FRET}}$, $-30$ mV | $2.9 \pm 0.6$ (24) | $3.8 \pm 0.8$ (15) | $7.0 \pm 0.9$ (19) |
| $I_{\text{FRET}}$, $-20$ mV | $2.6 \pm 0.7$ (24) | $3.9 \pm 0.8$ (15) | $7.6 \pm 0.9$ (19) |
| $I_{\text{FRET}}$, $-10$ mV | $2.8 \pm 0.8$ (24) | $3.8 \pm 0.8$ (15) | $8.3 \pm 1.4$ (19) |

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**CREB-dependent Transcription Activation**—The plasmids YKIDN and KIXCN coding for KID and KIX domains (25), respectively, were co-expressed (1:1) in COS1 cells with other cDNAs at 2:5:1 ratio to cDNA for $\alpha_{1C}$ subunit. To monitor transcriptional activation under voltage clamp conditions, we used the perforated patch clamp technique (26). $\beta$-Esin (20 $\mu$m, Sigma) was added to the pipette solution containing potassium gluconate 120, NaCl 10, MgATP 2.5, HEPES 5, and KCl 20; pH 7.2. External solution contained (in mM) NaCl, 140; KCl, 5.4, MgCl$_{2}$ 1, HEPES 5, CaCl$_{2}$ 2.0, and glucose 5.5; pH 7.4. Depolarization to $+20$ mV from the holding potential of $-90$ mV was applied to elicit maximum Ca$^{2+}$ current. To monitor changes of free Ca$^{2+}$ concentration, cells were loaded with Fluo-4 by incubation for 20 min at 37°C in 5 $\mu$m Fluo-4 AM (Molecular Probes) added to the external solution. For de-esterification of the probe, cells were incubated for another 20 min before experiment. Ca$^{2+}$ measurements were performed at 20–22°C using confocal microscope PCM2000 (Nikon, Inc.) with excitation by an argon laser at 488 nm and recording of the fluorescence emission at $>515$ nm.
Comparison of electrophysiological properties of the wild-type $\alpha_{1C}$ (A) and fluorescent labeled (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ (B), and (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ (C) $\text{Ca}^{2+}$ channels

The $\alpha_{1C}$ subunits were co-expressed with $\beta_2$ and $\alpha_6$ accessory subunits. $\text{Ba}^{2+}$ currents were elicited by 600-ms test pulses to $+10$ mV from a holding potential of $-90$ mV. The bath medium contained 20 mM $\text{Ba}^{2+}$. The time constants of the $\text{Ba}^{2+}$ current inactivation $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ were determined by two-exponential fitting. The approximated $\tau_{\text{slow}}$ values are presented solely to reflect the fact that slow inactivation is completely inhibited in the labeled $\alpha_{1C,77'}$ channel. $V_{\text{reset}}$, voltage for the peak current, and $V_{\text{reset}}$ voltage at 50% of the $\text{Ba}^{2+}$ current activation were determined from the current-voltage relationship. $V_{\text{reset}}$, the voltage at half-maximum of inactivation, and $I_{\text{reset}}$, fraction of non-inactivating current, were determined from the fitting of steady-state inactivation curves by Boltzmann function. Steady-state inactivation curves were measured using a two-step voltage clamp protocol. A 1-s conditioning pulse was applied at 30-s intervals with 10-mV increments from the holding potential $V_h = -90$ mV followed by a 250-ms test pulse to $+10$ mV. Peak current amplitudes were normalized to maximum value. All fittings were performed according to Ref. 33. Number of tested cells is shown in parentheses.

| $\alpha_{1C}$ | $V_{\text{max}}$ | $V_{\text{reset}}$ | $V_{50}\text{in}$ | Fraction $I_{\text{reset}}$ | $\tau_{\text{fast}}$ | $\tau_{\text{slow}}$ |
|---|---|---|---|---|---|---|
| A | $10.5 \pm 1.6$ (3) | $-12.5 \pm 1.7$ (3) | $-21.0 \pm 0.8$ (3) | $0.12 \pm 0.02$ (3) | $58.5 \pm 7.5$ (5) | $345 \pm 84$ (5) |
| B | $12.0 \pm 1.8$ (6) | $-11.0 \pm 1.6$ (6) | $-18.0 \pm 0.8$ (5) | $0.07 \pm 0.02$ (3) | $46.6 \pm 10.0$ (12) | $364 \pm 82$ (12) |
| C | $4.0 \pm 2.5$ (8) | $-15.5 \pm 1.8$ (6) | $-24.5 \pm 1.4$ (3) | $0.50 \pm 0.09$ (3) | $12.9 \pm 0.8$ (12) | --- |

![Fig. 1. Direct FRET measurements in the (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ channels.](image)

Channel (Fig. 2A) showed that the sequential transitions between the resting ($-90$ mV, Fig. 2A, c) and the inactivated states of the channel ($+40$ mV, Fig. 2A, b) produced a fully reversible increase in FRET: $I_{\text{FRET}}^{+40 \text{mV}}/I_{\text{FRET}}^{-90 \text{mV}} = 2.9 \pm 0.7$ ($n = 24$). The steady state inactivation analysis indicated that $\sim 7\%$ of the $\text{Ba}^{2+}$-conducting (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ channels remain available (Table II), suggesting that $\approx 93\%$ of the channels are inactivated and $\approx 7\%$ remain in the resting closed state at the end of the depolarization pulse shown in Fig. 2A. Since the resting state is characterized by low FRET, this fraction has only slightly contributed to the measurement of FRET. Taken together, these data indicate that the cytoplasmic tails of the channel in the inactivated state are rearranged into a conformation that gives greater FRET as compared with the closed resting state.

The electrophysiological properties of the $\alpha_{1C}$ channel are not significantly changed by the deletion of the N-terminal tail (30). In contrast, the proximal half of the C-terminal tail is essential for channel function (13, 30, 31). A fusion of ECFP to the full size, 662-amino acid C-terminal tail of $\alpha_{1C,77'}$ did not alter the electrophysiological properties of the (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ channel (data not shown), but reduced efficiency of FRET determined in the acceptor photobleaching assay to $0.19 \pm 0.02$ ($n = 29$), thus indicating that apparent separation of the fluorophores has increased. However, the intensity of FRET in the inactivated state ($+40$ mV) of this full-length (EYFP)$_N$-$\alpha_{1C,77'}$-(ECFP)$_C$ channel was not significantly different as compared with the truncated channel.

Voltage-dependent FRET in the Conducting State of the $\text{Ca}_{1.2}$ Channel—Rapid spontaneous channel inactivation complicates direct FRET imaging of the channel in the transient conducting state. Replacement of the cytoplasmic accessory $\beta_2$ subunit with $\beta_2$ (32) significantly slowed inactivation (compare $\text{Ba}^{2+}$ current traces on $d$ in Fig. 2, A and B) and thus
allowed the channel to be maintained in a predominantly conducting state. Voltage-dependent FRET was measured with the (EYFP)N−αIC,77−(ECFP)C channel at the end of a 3-s depolarization at +40 mV. Under these conditions, the Ba2+ current activation was almost maximal, whereas inactivation was minimal with respect to the time of FRET acquisition (marked by the red bar above the current traces). The voltage-dependent increase of FRET (3.8 ± 0.8, n = 15, see Fig. 2B) resulting from the transition of the channels from the resting (−90 mV) to a predominantly conducting state (+40 mV) was found not to be significantly different from that determined for the inactivated state (2.9 ± 0.7, Fig. 2A). Thus, the folding of the channel tails characterized by FRET in the ensemble of predominantly conducting channels was on average closer to
those determined for the inactivated than those determined for the resting state of the same channel.

This finding was independently confirmed by the study of the \( \alpha_{1C,IS-IV} \) channel that is deprived of slow inactivation by mutations introduced in the pore region (14). The (EYFP)\(_{N}\)-\( \alpha_{1C,77} \) (PH-ECFP)\(_{C} \) channel expressed in COS1 cells showed the characteristic sustained \( \text{Ba}^{2+} \) current (Fig. 2C, d). The amplitude of the \( \text{Ba}^{2+} \) current through the \( \alpha_{1C,IS-IV} \) channel was smaller than those through the \( \alpha_{1C,77} \) channel. Similar changes were observed earlier for other isoforms of the \( \text{Ca}_{\text{2.2}} \) channel with impaired \( \text{Ca}^{2+} \)-induced inactivation (33). This was found to be due to a lower open probability and a 10–15% reduction in single channel conductance (13). In the stable conducting (+40 mV) state of the \( \alpha_{1C,IS-IV} \) channel (Fig. 2C, b), the corrected

**Fig. 3.** Effect of immobilization and release of the C-terminal tail of \( \text{Ca}^{2+} \) channel on state-dependent FRET. A, direct FRET imaging. Phase contrast (a) and FRET images of COS1 cell expressing the (EYFP)\(_{N}\)-\( \alpha_{1C,77} \) (PH-ECFP)\(_{C} \) channel before (b) and after (c) release of the membrane-anchoring PH domain. Upper panels, digitally magnified image areas pointed by arrows. d, ratio of images (c and b) showing increased FRET in the membrane region in response to the C-tail release. Upper panel, three-dimensional image. B, inhibition of inactivation and state-dependent FRET in cell expressing the (EYFP)\(_{N}\)-\( \alpha_{1C,77} \) (PH-ECFP)\(_{C} \) channel with the membrane-trapped C-terminal tail. a, phase-contrast image of the expressing cell and schematic diagram of the \( \alpha_{1C} \) channel construct (below). b, the \( \text{Ba}^{2+} \) current trace elicited by depolarization to +40 mV from \( V_h = -90 \) mV. c and d, ratio of corrected FRET images recorded at the indicated membrane potentials. C, restoration of inactivation and state-dependent FRET after release of the C-tail. a, schematic diagram of the \( \alpha_{1C} \) channel construct. b, \( \text{Ba}^{2+} \) current trace elicited by depolarization to +40 mV from \( V_h = -90 \) mV. c and d, ratio of corrected FRET images recorded at the indicated potentials. Left panels, digital magnifications of the membrane region. Low panels, corresponding three-dimensional images. Scale bars, 8 \( \mu \)m. Voltage protocol was similar to Fig. 2.
**Fig. 4. CREB-dependent activation of transcription.** CREB activation was examined under perforated patch conditions by FRET between the EYFP-labeled KID domain of CREB and ECFP-labeled KIX domain of CREB-binding protein as interaction partners. A, CREB activation in COS1 cells expressing M1AChR and the (EYFP)N/H9251C/H177-(PH-ECFP)C channel with the membrane-trapped C-tail, before (a) and after (b and c) its release by ACh activation of M1AChR. d, representative trace of the Ca2+ current (20 mM Ca2+ in bath medium) showing the sustained component of Ca2+ conductance due to the C-tail anchoring, which was completely eliminated by the C-tail release (not shown). The CREB activation (A, c), shown by intensive FRET signal in the nucleus, was abolished (B) by the co-expression of the Ca2+-insensitive analogue of CaM (36) (CaM1234) or...
FRET increased 7.4 ± 0.9-fold (n = 19; p < 0.0005, compared with Fig. 2A, b) suggesting further reduction in the apparent distance between the labeled tails and/or dipole reorientation of the fluorophores (12) leading to an increase of FRET as compared with the resting state (~90 mV, see Fig. 2C, c). Taken together, these data point to distinct rearrangements of the α1C tails associated with voltage gating.

Role of the Voltage-gated Mobility of the C-terminal Tail in the Ca_{1.2} Channel Inactivation—To investigate a regulatory role of the voltage-gated rearrangements of the α_{1C} C-terminal tail, the tail was anchored to the plasma membrane via the PH domain of phospholipase Cδ1 (Fig. 3). This domain binds specifically to phosphatidylinositol bisphosphate in the inner leaflet of the plasma membrane. Hydrolysis of phosphatidylinositol bisphosphate by activation of phospholipase C induces redistribution of the PH domain to the cytoplasm (15, 34).

The (EYFP)_{N}-α_{1C,771}-(PH-ECFP)_{C} channel with anchored C-tail was co-expressed in COS1 cells with epidermal growth factor (EGF) receptor to permit a release of the PH-tagged C-terminal tail by activating phospholipase γ and phosphatidylinositol bisphosphate hydrolysis in response to exposure of the cell to EGF (Fig. 3A). The Ba^{2+} current through the channel with the anchored C-terminal tail was activated in the characteristic range of membrane potentials but exhibited a very slowly inactivating component of the Ba^{2+} current (Fig. 3B, b). Membrane trapping of the N-terminal tail did not alter the inactivation properties of the channel (data not shown).

Very little, if any, FRET was observed with the anchored C-terminal tail in both the conducting (Fig. 3B, b) and the resting (d) states of the channel. The release of the tail by EGF treatment irreversibly restored the ability of the channel to inactivate fully as can be seen from the complete decay of the current (Fig. 3C, b) and increased FRET in the inactivated state (Fig. 3C, c). The ratio (IFRET_{−90mV}/IFRET_{+90mV}) = 1.9 ± 0.9 (n = 5, p < 0.05) was not different to that observed with the (EYFP)_{N}-α_{1C,771}-(ECFP)_{C} (Fig. 2A) and (EYFP)_{N}-α_{1C,771}-(ECFP)_{C} (not shown) channels. Thus, limitations imposed on free movement of the C-terminal tail of the α_{1C} channel affect inactivation properties as well as the associated FRET. When released, the C-tail appears to assume a functional conformation as determined by both the return of the normal inactivation properties of the channel and voltage-dependent FRET. This precludes a re-insertion of the PH domain into the membrane. Even a 30-min washout period, the longest that we were able to achieve without losing the voltage clamp, did not restore the membrane association of the PH domain distinguished by the properties of the channel with the trapped tail.

Role of the Voltage-gated Mobility of the C-terminal Tail in Regulation of CREB-dependent Transcription—The amplitude of the voltage-gated mobility of the Ca_{1.2} channel C-terminal tail may be sufficient to have a role in Ca^{2+} signal transduction. It has been shown previously (8) that Ca_{1.2} channels are important for Ca^{2+}-induced activation of cAMP-responsive element-binding protein (CREB)-dependent transcription. In this work, to study the role of the voltage-gated mobility of the Ca^{2+} channel C-terminal tail for transcriptional activation, we investigated the interaction between KID and KIX domains of CREB and co-activator CREB-binding protein under voltage clamp conditions by monitoring FRET between (EYFP)-KID and (ECFP)-KIX, both containing nuclear localization sequences (25). Use of perforated patch clamp technique (26) allowed us to preserve the integrity of the cytoplasmic content of the cell, crucial in retaining components of signaling cascade involved in CREB-dependent transcription. We found that when the C-terminal tail of the α_{1C,771}-(PH)_{C} channel was anchored via the PH domain to the plasma membrane (Fig. 4A, a), activation of the channel by depolarization to +20 mV increased intracellular free Ca^{2+} concentration ([Ca^{2+}]_{i}) detected by the free Ca^{2+} indicator Fluo-4 (Fig. 4A, a, see image on the lower panel). However, stimulation of the sustained Ca^{2+} conductance of the anchored channel by depolarization (Fig. 4A, d) did not cause strong activation of CREB-dependent transcription despite the presence of the CaM-binding IQ motif of the C-tail 1624–1635 (35), previously identified as important for CREB activation (8). Release of the C-terminal tail of the channel, induced at −90 mV by ACh stimulation of the co-expressed type 1 muscarinic acetylcholine receptor (M1AcR), was accompanied by activation of the inositol 1,4,5-trisphosphate-dependent Ca^{2+} release but did not induce substantial activation of CREB-dependent transcription (Fig. 4A, b). It was the release of the C-terminal tail of the channel combined with the activation of Ca^{2+} conductance by depolarization that was essential to re-establish the voltage-gated signaling sufficient for CREB-dependent transcription activation (Fig. 4A, c). The fact that this response was inhibited by co-expression of Cam1234 (Fig. 4B), a Ca^{2+}-insensitive analogue of CaM retaining the affinity to CaM-binding sites (36), points to a critical role of CaM in signal transduction by the voltage-gated mobile C-terminal tail of the α_{1C} channel. Selective disruption of the apo-CaM-binding site 1572–1598 (37–39) on the C-terminal tail of the α_{1C} channel (33) (Fig. 4C) abolished activation of CREB-dependent transcription despite unrestricted mobility of the C-terminal tail and the presence of CaM and IQ motif (8). These results, for the first time, demonstrated the role of the Ca^{2+} channel C-tail as a voltage-gated mobile carrier of the signal for CREB-dependent transcriptional activation (Fig. 4D). This signal appears to be associated with apo-CaM binding to the C-tail and may involve its Ca^{2+} loading (9) and translocation to the IQ motif (8, 36), a Ca^{2+}-filled CaM-binding site on the C-tail.

DISCUSSION

In this report, we provide strong evidence that the C-terminal cytoplasmic tail is a functionally important moving part of the voltage-gated Ca^{2+} channel. Previously implicated as cytoplasmic channel elements that respond to voltage gating were the cytoplasmic domain between repeat II–III of the skeletal muscle α_{1S} subunit (40), inactivation gates of Na_{v} channel (41), and the ball-and-chain (N-type) inactivation determinant of K^{+} channel (7, 42). The state dependence of FRET in the α_{1C} channel is associated with conformational refolding of the cytoplasmic parts in the resting, conducting, and inactivated states. FRET increased upon transition from the resting to the inactivated or conducting states of the Ca^{2+} channel suggesting gating-dependent conformational mobility of the channel tails. The anchoring of the C-terminal tail to the plasma mem-
brane impaired both the channel inactivation and FRET until fully recovered with its release. Thus, the state-dependent mobility of the cytoplasmic C-terminal tail is essential for regulation of the Ca$^{2+}$ channel.

In addition to relative proximity of the tagged α1C tails, their angular orientation may contribute to FRET because the environment of the fluorophores in the (EYFP)$_{α1C}$-(ECFP)$_{α1C}$ channels may be structured and their segmental motions may not independently randomize the orientations ($\chi^2 < 2/3$) (12). The $\chi^2$ factor adds to the uncertainties complicating interpretation of the FRET measurements in terms of translational distances and is of particular concern when dipoles become oriented perpendicular to one another ($\chi^2 = 0$). Because FRET was consistently observed, such perpendicular orientation seems unlikely. In fact the corresponding voltage-dependent movement of the α1C cytoplasmic C-tail is sufficient to reach other targets and be involved in signal transduction. An important regulatory signal associated with the C-terminal tail is CaM, which supports Ca$^{2+}$-dependent inactivation of the Ca$^{2+}$ channel (35, 37, 38). It is possible that the C-terminal tail mobility may, in a state-dependent manner, provide a coordinated transfer of CaM between the channel pore inner mouth, where it is loaded in a state-dependent manner, provide a coordinated transfer of CaM-activated protein kinase (8), where Ca$^{2+}$/CaM targets and be involved in signal transduction. An important cave in the Nature of the Ca$^{2+}$ channel.

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