Ca\(_v\)\(\beta\)-subunit displacement is a key step to induce the reluctant state of P/Q calcium channels by direct G protein regulation

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P/Q Ca\(^{2+}\) channel activity is inhibited by G protein-coupled receptor activation. Channel inhibition requires a direct G\(\beta\)\(\gamma\) binding onto the pore-forming subunit, Ca\(_{2,1}\). It is characterized by biophysical changes, including current amplitude reduction, activation kinetic slowing, and an I-V curve shift, which leads to a reluctant mode. Here, we have characterized the contribution of the auxiliary \(\beta\_3\)-subunit to channel regulation by G proteins. The shift in I-V to a P/Q reluctant mode is exclusively observed in the presence of \(\beta_3\). Along with the observation that G\(\beta\)\(\gamma\) has no effect on the I-V curve of Ca\(_{2,1}\) alone, we propose that the reluctant mode promoted by G\(\beta\)\(\gamma\) corresponds to a state in which the \(\beta_3\)-subunit has been displaced from its channel-binding site. We validate this hypothesis with a \(\beta_3\)-I-H\(_{2,1}\) loop chimera construct. G\(\beta\)\(\gamma\) binding onto the I-H\(_{2,1}\) loop portion of the chimera releases the \(\beta_3\)-binding domain and makes it available for binding onto the I-II loop of Ca\(_{2,1}\), a G protein-insensitive channel. This finding is extended to the full-length Ca\(_{2,1}\) channel by using fluorescence resonance energy transfer. G\(\beta\)\(\gamma\) injection into Xenopus oocytes displaces a Cy3-labeled \(\beta_3\)-subunit from a GFP-tagged Ca\(_{2,1}\) channel. We conclude that \(\beta\_3\)-subunit dissociation from the channel complex constitutes a key step in P/Q calcium channel regulation by G proteins that underlies the reluctant state and is an important process for modulating neurotransmission through G protein-coupled receptors.

Materials and Methods

Materials. Purified bovine brain G\(\beta\)\(\gamma\) was from Calbiochem and was used as described (9). Collagenase IA was from Sigma. The GST-AID\(_2\) fusion protein and the GFP-Ca\(_{2,1}\) fusion construct have been described (5, 17). For GFP-Ca\(_{2,1}\), the N terminus of Ca\(_{2,1}\) was fused to the C terminus of a modified version of GFP (mutations S65T for increased brightness and optimized spectral properties; and V163A, I167T, S175G for better thermostability).

Fluorescence Resonance Energy Transfer (FRET) Measurements. Albino oocytes were analyzed with a \(\times 10\) objective by confocal microscopy using the XY mode of fluorescence collection of the TCS-SP2 software (Leica, Heidelberg) 4–7 days after injection. The fluorescence was measured through 14 pass-band filters (10 nm width) to reconstruct the emission spectrum. For each measurement, the entire region of the plasma membrane at the equatorial level was considered for the fluorescence intensity. This intensity was normalized to the area of surface analyzed. The FRET levels were estimated as the ratio (575/515) of the fluorescence at 575 nm (emission peak of the acceptor: Cy3) versus the fluorescence at 515 nm (emission peak of the donor: GFP). After injection of G\(\beta\)\(\gamma\) into oocytes, fluorescence scans were rapidly monitored at various times. The depression, observed \(\sim\)545 nm in the spectra of the XY-L experiments, is due to a physical displacement of the \(\beta\_3\)-subunit from its binding site on Ca\(_{2,1}\).

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Abbreviations: FRET, fluorescence resonance energy transfer; FRETN, normalized FRET; W, willing; R, reluctant; HI, heat-inactivated.

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to the use of a triple dichroic mirror (488, 545, and 633 nm). This depression can be avoided by using a neutral 30/70 filter instead of a dichroic mirror. However, the poor reflection of the 488-nm excitation wavelength resulting from the use of this filter gives rise to spectra of much lower dynamics. Altogether, the use of the dichroic mirror provides higher dynamics to our spectra and does not perturb FRET estimation at a 575/515 fluorescence ratio, thereby increasing measurement reliability. Background spectra from control noninjected oocytes were subtracted from spectra from injected cells.

Further information on Western blotting, chimera constructions, in vitro translation and GST pulldown assays, His-β3 purification and Cy3 labeling, and Xenopus oocyte injection and electrophysiological recording can be found in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Results

β3-Subunit Expression Is Required for Some Important Biophysical Effects of Gβγ. We first confirmed by immunocytochemistry that the Xenopus oocyte β3-subunit, which has high homology with the rat sequence (18), was undetectable at the protein level; a condition necessary for the correct interpretation of our data (Fig. 1a). We next investigated the biophysical effects of Gβγ on Ca2,1 in the absence and presence of β3 of cRNA origin, to determine the contribution of β3 to Gβγ regulation. Out of β3 and β2, two β3-subunits known to bind the P/Q channel, β3 was used because it is known to interact only with AID (19). Injection of 5 ng of Gβγ in Xenopus oocytes expressing Ca2,1 alone produces a significant inhibition of current amplitude at 0 mV (50 ± 3%, n = 5), illustrating the β-independent effects (Fig. 1b). When β3 is coexpressed, Gβγ produces an even greater inhibition of Ca2,1 current amplitude (up to 81 ± 9% at 0 mV, n = 8). An average pre-pulse facilitation of 8% was observed, probably reflecting a partial dissociation of Gβγ from this rabbit Ca2,1 isoform. In both conditions, injection of heat-denatured Gβγ was without effect on current amplitude (Fig. 1c). Current kinetics (activation and inactivation) after the effect of Gβγ is indistinguishable whether or not β3 is expressed (Fig. 1d).

Average times to peak of the currents after Gβγ effect were 28.8 ± 8.1 ms (−β3, n = 8) and 28.7 ± 4.1 ms (+β3, n = 10). As interpreted by others (20), these results could suggest that β3 promotes G protein regulation. Alternatively, the following points suggest that this finding results from a displacement of the β3 from Ca2,1: (i) in the absence of β3, Gβγ has no effect on either the I-V curve (Fig. 2a) or the steady-state inactivation properties (Fig. 2b) of Ca2,1; (ii) β3-subunit is known to displace, toward hyperpolarizing potentials, the I-V curve (−19.5 mV, n = 13) and the steady-state inactivation properties of the channel (−28.9 mV, n = 12; Fig. 2c and d); and (iii) acute injection of Gβγ reverses the shifting effects of β3 on both the I-V curve (+19.2 mV, n = 18) and the steady-state inactivation (+25.3 mV, n = 10; Fig. 2c and d). Thus, this reversal in the shift of the I-V curve translates in an apparent extra inhibition (apparent promoting effect of β3) when a 0 mV pulse is used to activate the channel (Fig. 1b).

Working Hypothesis. Assuming that Gβγ displaces β3 from its binding site, then the final inhibited state of Ca2,1 should be the same, independent of the presence of β3 (see Fig. 6, which is

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published as supporting information on the PNAS web site). For the sake of clarity, let us conceptually dissociate the dual effects of the binding of $\gamma$ on Cav$_{a/\beta}$ into two concomitant effects: (i) the displacement of $\beta$ from Cav$_a$ (the focus of this study), and (ii) the inhibition of Cav$_a$ alone by G$\beta$$\gamma$. If our molecular hypothesis is correct, then the following equation (see Supporting Materials and Methods for equation derivation) should allow a theoretical estimation of the apparent promoting effect of $\beta_3$ on the inhibition of Cav$_{2.1}$ current induced by G$\beta$$\gamma$ at a given test potential.

$$Inh_{\beta_3} = \frac{\phi - 1}{\phi}$$

where $\phi$ represents the change in normalized conductance of the channel produced by $\beta$-subunit dissociation. At 0 mV, the theoretical value of G$\beta$$\gamma$ inhibition in the presence of a virtual $\beta_3$-subunit (mean 87 ± 1%, n = 5) is in close agreement with the experimental mean value for Cav$_{2.1/\beta_3}$ channels (81 ± 9%, n = 8; Fig. 6 d and e). In contrast, the “$\beta$-promoting effect” is clearly lost at 30 mV, as verified both experimentally and after modeling. Experimentally, the equal inhibition levels observed at 30 mV for the Cav$_{2.1}$ and Cav$_{2.1/\beta_3}$ channels, is further indication that $\beta$-subunit dissociation has probably no significant effect on Cav$_{2.1}$ channel properties, other than the depolarizing shift in the I-V curve. The apparent extra inhibition produced by G$\beta$$\gamma$ at 0 mV, which is observed in the presence of $\beta_3$, may well result from a displacement of this auxiliary subunit from the channel. We thus propose that binding of G$\beta$$\gamma$ complex onto Cav$_{2.1}$ produces two distinct functional effects: (i) a $\beta$-independent current amplitude inhibition, and (ii) a $\beta$-dependent shift in the I-V curve and steady-state inactivation voltage-dependencies of the channel. In the following experiments, we shall focus our study on the mechanisms of the $\beta$-dependent effects of G$\beta$$\gamma$ regulation.

**Structural Rationale.** It is now widely accepted that the AID motif, present on the I-II loop, constitutes the main binding site for $\beta$-subunit dissociation. At 0 mV, the full-length I-II loop of the Cav$_{2.1}$ family also contains multiple binding sites for G$\beta$$\gamma$, and hence it is correct, then the following equation (see Supporting Materials and Methods for equation derivation) should allow a theoretical estimation of the apparent promoting effect of $\beta_3$ on the inhibition of Cav$_{2.1}$ current induced by G$\beta$$\gamma$ at a given test potential.

$$Inh_{\beta_3} = \frac{\phi - 1}{\phi}$$

where $\phi$ represents the change in normalized conductance of the channel produced by $\beta$-subunit dissociation. At 0 mV, the theoretical value of G$\beta$$\gamma$ inhibition in the presence of a virtual $\beta_3$-subunit (mean 87 ± 1%, n = 5) is in close agreement with the experimental mean value for Cav$_{2.1/\beta_3}$ channels (81 ± 9%, n = 8; Fig. 6 d and e). In contrast, the “$\beta$-promoting effect” is clearly lost at 30 mV, as verified both experimentally and after modeling. Experimentally, the equal inhibition levels observed at 30 mV for the Cav$_{2.1}$ and Cav$_{2.1/\beta_3}$ channels, is further indication that $\beta$-subunit dissociation has probably no significant effect on Cav$_{2.1}$ channel properties, other than the depolarizing shift in the I-V curve. The apparent extra inhibition produced by G$\beta$$\gamma$ at 0 mV, which is observed in the presence of $\beta_3$, may well result from a displacement of this auxiliary subunit from the channel. We thus propose that binding of G$\beta$$\gamma$ complex onto Cav$_{2.1}$ produces two distinct functional effects: (i) a $\beta$-independent current amplitude inhibition, and (ii) a $\beta$-dependent shift in the I-V curve and steady-state inactivation voltage-dependencies of the channel. In the following experiments, we shall focus our study on the mechanisms of the $\beta$-dependent effects of G$\beta$$\gamma$ regulation.

**Effect of G$\beta$$\gamma$ on the Conformation of a Chimeric $\beta_3$-I-II$_{2.1}$ Protein.** We used a molecular in vitro strategy to test the nature of the interactions between Cav$_{2.1}$, $\beta_3$, and G$\beta$$\gamma$. First, we designed a chimera molecule between $\beta_3$ and the I-II loop of Cav$_{2.1}$ (Cav$_{3-I-II_{2.1}}$) with the expectation that the AID domain from I-II$_{2.1}$ recognizes its binding site on $\beta_3$ [BID site (22)]. Such a strategy simplifies the number of molecular partners used in G$\beta$$\gamma$ competition experiments and forces a stoichiometry of one $\beta_3$ molecule for one I-II loop sequence. Also, the limited amount of translated chimera molecule is easy to saturate by G$\beta$$\gamma$ binding onto the I-II$_{2.1}$ part. Fig. 3b–e summarizes the experiments that lead us to conclude that G$\beta$$\gamma$ association on the I-II$_{2.1}$ part of the molecule results in the dissociation of $\beta_3$ from AID. A GST protein fused to an enlarged AID sequence of Cav$_{2.1}$ (GST-AID$_{2.1}$) (5) binds in vitro-translated [35S]$\beta_3$-subunit (Fig. 3b). On the other hand, fusion of $\beta_3$ to the I-II loop of Cav$_{2.1}$ prevents its interaction with GST-AID$_{2.1}$ (Fig. 3c). To demonstrate that this result is due to an intramolecular interaction between BID and AID$_{2.1}$, we deleted the 18-aa AID$_{2.1}$ residues. This deletion restores the ability of the chimera to bind onto GST-AID$_{1.2}$ (Fig. 3d). Evaluating the binding of $\beta_3$-I-II$_{2.1}$ onto GST-AID$_{1.2}$ represents a good test to identify inhibitors of the internal AID$_{2.1}$/BID interaction. A molecule will induce the binding of the chimera protein only if it displaces the internal AID$_{2.1}$/BID interaction without affecting the subsequent BID/ AID$_{2.1}$ interaction. As shown in Fig. 3c, G$\beta$$\gamma$ is precisely this type of inhibitor, because preincubation of increasing concentrations of purified G$\beta$$\gamma$ with [35S]$\beta_3$-I-II$_{2.1}$ (30 min at 20°C) induces its binding to GST-AID$_{1.2}$. The use of GST-AID$_{1.2}$ here appears to be beneficial because, contrary to Cav$_{2.1}$, Cav$_{1.2}$, a member of the L-type family, is unable to bind G$\beta$$\gamma$ (12). The displacement of AID$_{2.1}$ from its BID-binding site occurs with an EC$_{50}$ of 121 nM for G$\beta$$\gamma$. This value is in close agreement with the reported dissociation constant of G$\beta$$\gamma$ for the I-II$_{2.1}$ loop (12) and compares well with the effective concentrations observed in other physiological systems (23). These observations were confirmed in an ex vivo context by evaluating the increase of Cav$_{1.2}$ current density induced by the unfolding of $\beta_3$-I-II$_{2.1}$ by G$\beta$$\gamma$ (see Supporting Materials and Methods, and Fig. 7, which is published as supporting information on the PNAS web site).

**Dynamic Observation of a G$\beta$$\gamma$-Induced $\beta$ Displacement by FRET in Single Living Cells.** To follow the dynamic of the interaction between the full-length Cav$_{2.1}$- and $\beta$-subunits in a cellular context, we
designed a FRET strategy. In our case, we favored the combination of GFP-Ca$_{2.1}$ and chemically labeled Cy3-$\beta_3$, which constitutes a favorable Förster donor/acceptor pair (Förster radius of 60 Å (24)). We demonstrated that (i) $\beta_3$ can be labeled with Cy3-maleimide, and (ii) remains functional after injection in *Xenopus* oocytes despite the labeling (see Supporting Materials and Methods, and Fig. 8, which is provided as supporting information on the PNAS web site). Of note, oocytes have optical properties that make fluorescence studies on living cells valid, only in the vicinity of the plasma membrane. Indeed, the filter effect of the cytoplasm of oocytes in *Xenopus* remains functional after injection in oocytes (Fig. 4a). Although living oocytes should not be used to study subcellular protein distribution with a confocal microscope, their use remains perfectly valid for fluorescence emission studies in the vicinity of the plasma membrane. We first showed that noninjected albino cells emitted very low levels of fluorescence in our experimental conditions (Fig. 4e). Injection of either Cy3-$\beta_3$ protein or GFP-Ca$_{2.1}$ CDNA, alone or in combination, results in strong fluorescence signals in the vicinity of the plasma membrane (Fig. 4e). Fluorescence of GFP-Ca$_{2.1}$ alone is indicative of the expression of the channel at the plasma membrane in the absence of Cy3-$\beta_3$, which is in agreement with biophysical data (Figs. 1b and 2a and b). The more diffuse distribution of Cy3-$\beta_3$ most likely reflects the cytoplasmic distribution of the protein. We also noted that the apparent distribution of GFP-Ca$_{2.1}$ fluorescence in the presence of unlabeled $\beta_3$, or Cy3-$\beta_3$ fluorescence in the presence of unlabeled Ca$_{2.1}$, was not modified (not shown). According to our experimental conditions, Cy3-$\beta_3$ was in molar excess compared with GFP-Ca$_{2.1}$.

Fig. 4b represents average emission spectra from cells in four experimental conditions (noninjected, injected with GFP-Ca$_{2.1}$ plus unlabeled $\beta_3$, injected with labeled Ca$_{2.1}$ plus Cy3-$\beta_3$, and injected with GFP-Ca$_{2.1}$ plus Cy3-$\beta_3$). The fluorochromes were excited at 488 nm and the emission spectra recorded from 500 to 640 nm with 14 successive 10-nm-wide band paths. The settings (laser power and photomultiplier gain) of the confocal microscope were kept constant during all of the measurements. The fluorescence spectrum of the noninjected oocytes (dotted line) is very low, compared with injected oocytes, and was subtracted from the data shown in Fig. 5 and for FRET calculations. Under the same conditions, oocytes expressing GFP-Ca$_{2.1}$ plus unlabeled $\beta_3$ give a stronger fluorescence signal with a maximal emission at $\lambda_{max}$ of 515 nm (Fig. 4b, green line). Although the excitation wavelength at 488 nm limits the excitation of Cy3-$\beta_3$ ($\lambda_{max}$ at 543 nm), we observed a fluorescence emission spectra with a maximal emission at 575 nm for oocytes injected with 1.75 ng of Cy3-$\beta_3$ and expressing unlabeled Ca$_{2.1}$ (Fig. 4b, dark red line). The fluorescence intensity level of oocytes injected with Cy3-$\beta_3$ demonstrated a good reproducibility. Also, the absence of unlabeled Ca$_{2.1}$ did not alter the observed emission spectra of Cy3-$\beta_3$. When both GFP-Ca$_{2.1}$ and Cy3-$\beta_3$ are present in the oocyte (Fig. 4b, red line), the emission signal strongly decreases at 515 nm, whereas it increases significantly at 575 nm, suggesting that FRET might occur between these two molecules. This effect

**Fig. 5.** Analysis of G$\beta$y-induced P/Q calcium channel disassembly. (a) Average emission spectra and effect of the injection of 5 ng of G$\beta$y for oocytes in various experimental conditions: (Top) Unlabeled Ca$_{2.1}$ plus Cy3-$\beta_3$. (Middle) GFP-Ca$_{2.1}$ plus unlabeled $\beta_3$. (Bottom) GFP-Ca$_{2.1}$ plus Cy3-$\beta_3$, ○, noninjected; □, G$\beta$y-injected. (b) Box plot representation of FRETN values for noninjected G$\beta$y oocytes (●), G$\beta$y-injected (5 ng), and injection of 5 ng of Hi-G$\beta$y. (c) Kinetics of FRET decrease induced by 5 ng of G$\beta$y injection. Illustration of two representative cells. (d) Model of calcium channel regulation by the G$\beta$y complex. W, β-bound W state; R, G$\beta$y-bound R state; R', G$\beta$y-unbound R state.
is illustrated by the comparison of average emission spectra of oocytes expressing GFP-CaV2.1 and Cy3 -β3 with the sum of the average emission spectra of cells expressing GFP-CaV2.1 plus unlabelled β3 and of cells expressing unlabelled CaV2.1 plus Cy3-β3 (Fig. 5c, blue line). This summed spectrum should be representative of the theoretical spectrum for oocytes containing both GFP-CaV2.1 and Cy3-β3 if there was no FRET. Comparing the two spectra in Fig. 5c, the FRET occurring between GFP-CaV2.1 and Cy3-β3 can be evidenced by the decreased emission at 515 nm, and the increased emission at 575 nm.

Next, we analyzed the effect of injecting 5 ng of Gβγ onto the average emission spectrum of oocytes containing both GFP-CaV2.1 and Cy3-β3. As shown in Fig. 5a, injection of Gβγ induces a drastic modification of the emission spectrum. The observed changes (decreased emission at 575 nm, and increased emission at 515 nm) are coherent, with a reduction in FRET efficiency. This observation qualitatively validates the concept that Gβγ is able to dissociate Cy3-β3 from GFP-CaV2.1. A similar Gβγ injection has no effect on the fluorescence emission spectra of oocytes containing GFP-CaV2.1 plus unlabelled β3 or unlabelled CaV2.1 plus Cy3-β3 (Fig. 5a). To quantify precisely the FRET changes between GFP-CaV2.1 and β3-Cy3 and to normalize for fluorescence intensity variability of the fluorophores concentrations in different cells, we followed the method of Gordon et al. (25) for FRET calculation. By using the fluorescence recorded at 515 and 575 nm in our experimental conditions, the equation for normalized FRET (FRETN) calculations is:

\[
\text{FRETN} = \frac{[F] - D_f(D_f/D_d) - A_f(F_a/A_a)}{\left[\frac{G}{F_d} \times D_f \times A_f\right]}
\]

where \(F\) is the fluorescence at 575 nm of GFP-CaV2.1 plus Cy3-β3 (excitation at 488 nm); \(D_f\) is the fluorescence at 515 nm of GFP-CaV2.1 plus Cy3-β3 (excitation at 488 nm); \(D_d\) is the fluorescence at 515 nm of GFP-CaV2.1 alone (excitation at 488 nm); \(D_d\) is the fluorescence at 575 nm of GFP-CaV2.1 alone (excitation at 488 nm); \(A_f\) is the fluorescence at 575 nm of GFP-CaV2.1 plus Cy3-β3 (excitation at 543 nm); \(A_a\) is the fluorescence at 575 nm of unlabelled CaV2.1 plus Cy3-β3 (excitation at 488 nm); \(a\) is the fluorescent at 575 nm of unlabelled CaV2.1 plus Cy3-β3 (excitation at 543 nm); and \(G\) is a constant related to the respective transmissions of the three filter sets used (\(F, A, D\)) and to the quantum yield of the two fluorophores. As suggested (25), \(G\) will be set arbitrary to a value of 1. In this equation, the term \(-D_f(D_f/D_d)\) corresponds to the correction of FRET for donor fluorescence crosstalk (GFP), whereas the term \(-A_f(F_a/A_a)\) corresponds to the correction of FRET signal for acceptor fluorescence crosstalk (Cy3). These crosstalks can be observed in the individual cases presented in Fig. 4b (see arrows). Using the equation, we came up with an average FRETN (Gβγ) value of 0.0156 ± 0.0048 (n = 5) before injection of Gβγ (Fig. 5b). This value is decreased to FRETN(β-Gβγ) = -0.0012 ± 0.0013 (n = 5) 15 min after injection of 5 ng of heat-inactivated Gβγ (HIP-Gβγ) produced no decrease of FRETN [FRETN(HI-Gβγ) = 0.0224 ± 0.0095 (n = 3)]. In theory, a FRET decrease can be due to either a change of the relative orientation of the two fluorophores, or to an increase in the interfluorochrome distance. Because the orientation of the flexible Cy3-maleimide fluorochromes is randomly changing, a possible reorientation of GFP, induced by Gβγ interaction with CaV2.1 (maybe including its N terminus), should statistically not impact the FRET value very much. The presence of three different Cy3-maleimide fluorophores, located at distant sites on β3, all outside the interaction site with CaV2.1, further minimizes the chances that GFP reorientation should affect the FRET value to the extent we observe. Assuming a random relative orientation, the large Förster radius between GFP and Cy3 (60 Å) allows the observation of FRET for distances up to 100 Å between GFP and any one of the three Cy3 present on the β3-subunit. Therefore, the most likely explanation for the decrease in FRET, induced by Gβγ, should be a large increase in interfluorochrome distance, compatible with a dissociation of the GFP-CaV2.1/Cy3-β3 complex. Assuming that FRET decrease induced by Gβγ is truly due to the dissociation of Cy3-β3 from GFP-CaV2.1, then, as noticed earlier (25), FRETN is directly proportional to the concentration ratio [GFP-CaV2.1/Cy3-β3 complex]/[total GFP-CaV2.1] · [total Cy3-β3]. Due to the large molar excess of Cy3-β3 in oocytes, all of the GFP-CaV2.1 present at the plasma membrane can be considered to be associated with Cy3-β3 before injection of Gβγ. This assumption is validated by the biophysical studies (Figs. 2 and 8) and earlier studies (3). Furthermore, Gβγ has no effect on the total concentrations of Cy3-β3 and GFP-CaV2.1 in oocytes. Therefore, the FRETN value that decreases to 0 after Gβγ injection is indicative that Gβγ displaces all of the Cy3-β3 from GFP-CaV2.1 channels.

Our approach on living oocytes provided the possibility to monitor FRET changes at various times after Gβγ injection (Fig. 5c). FRETN decrease reached completion 3 min after Gβγ injection.

**Discussion**

Activation of G proteins results in the binding of Gβγ onto the I-II loop of CaV (9, 12) and mediates inhibition of presynaptic Ca2+ currents (7). The contribution of the β-subunit to the regulation of channel activity by Gβγ has never been clearly elucidated (11). Initially, it was thought that β antagonized the effect of G proteins (26). Recent studies (20) show, on the contrary, that β is required for the willing mode of the channel and promotes G protein regulation. Some of the discrepancies reported on the mechanisms of regulation by G proteins may simply stem from the fact that data based exclusively on biophysical approaches have been interpreted directly in molecular terms. As shown in this manuscript, some of these modulations are independent of the presence of the β-subunit. We purposely developed molecular approaches in parallel with biophysical studies to exclusively address the molecular mechanisms underlying the β-dependent Gβγ modulation.

**Evidence That Gβγ Displaces β from Its CaV-Binding Site**

Our approaches were developed to study the relationship between CaV2.1, β3, and Gβγ. The biophysical experiments were aimed at solving some of the contradictory reports from the literature. To avoid many drawbacks related to the activation of G protein-coupled receptors, we directly injected controlled amounts of purified Gβγ complex. The biophysical effects of Gβγ are not due to an indirect alteration of the phosphorylation state of CaV2.1 because we have similar results in the presence of 500 ng/ml staurosporin (data not shown). Modeling of the biophysical data suggests that Gβγ may functionally displace β3 from the channel. We aimed to know if this functional displacement was actually due to a physical removal of β3 from its unique binding site on CaV2.1 or an allosteric effect of Gβγ that prevented the β3 regulation. By using a pull-down assay, we first demonstrated that Gβγ disrupts the intramolecular interaction between both the AID2.1 and BID sites of the β3-I-III1 chimera. Recently, Herlitze et al. (28) came to an opposite conclusion. However, these authors used a much shorter fragment of the I-II loop that inconveniently misses several Gβγ-binding sites. Although Gβγ has the ability to bind to AID (12), we envision that interaction of Gβγ with these other sites should favor some kind of unzipping of β3 from AID. In addition, these authors omitted Gγ from their studies (28), which is worrisome, because we expect Gγ to play a key function in G protein regulation. Gγ has structural homology with the III-IV loop of CaV2.1 (29). Because this loop interacts with several critical AID residues of the I-II loop (29), we expect that Gγ contributes to β3-subunit displacement. Obviously, the precise submolecular sequence of events responsible for β3 dissociation from the AID site will require further dissection.

To extend these observations to the full-length CaV2.1-
β3-subunit in a cell context, we developed a FRET strategy. When Cy3-labeled β3 protein and GFP-Ca2.1 fusion protein were coinjected in oocytes, we observed a strong FRET signal resulting from their direct association. The dynamic decrease of FRET signal induced by Gβγ in this system gives a real-time demonstration of the displacement of Cy3-β3 from GFP-Ca2.1. Theoretically, a decrease of FRET can be interpreted as either a change in conformation of the GFP-Ca2.1/Cy3-β3 complex or a dissociation of Cy3-β3 from the channel. Assuming a random relative orientation, the large Förster radius of the GFP/Cy3 pair (R0 = 60 Å) allows the observation of FRET for distances up to 100 Å between GFP and any one of the three Cy3. For comparison, the theoretical Stokes radius of globular proteins like the β3-subunit is ~50 Å. Thus, the three Cy3 labels must contribute statistically to the FRET. The complete suppression of FRET induced by Gβγ (as estimated from FRETN) indicates that β3 should be displaced by a distance greater than its own Stokes radius. Of course, it cannot be completely excluded that this FRET suppression results from a simple reorientation of the respective emission and excitation dipolar moments of the fluorophores. Reorientation of the GFP fluorophore is possible because this rigid tag is attached to the N terminus of Ca2.1, a possible binding site of Gβγ. However, three flexible Cy3 acceptor fluorophores located at different locations on β3 should randomize the FRET signal, no matter what orientation GFP or possible binding site of Gβγ/H9252/H9253 produces a simple swinging of β3 by favoring its attachment onto a different site, because the I-II loop is the only binding domain of this β isoform. Finally, although the allostERIC model defended by others (28) cannot be excluded only on the basis of the present FRET experiment, the convergence of these data with those obtained here by the other experimental approaches gives a solid support to the hypothesis of a physical dissociation of the Ca2-β heterodimer by Gβγ.

The Shift of the Channel from a W State to an R State Results from the β Displacement. It was initially reported that the neurotransmitter-induced inhibition of neuronal calcium currents results mainly from a change in channel voltage dependence (10). Bean (10) proposed that calcium channels can shift from a W state, characterized by a low-voltage activation potential, to an R state requiring stronger depolarization for activation. This shift can be induced by the binding of Gβγ onto the channel (11). In the absence of β3, we did not observe any effect of Gβγ on the channel’s voltage dependence of activation. On the other hand, the same experiment performed with β3 resulted in an important shift of the voltage dependence of activation. This biophysical observation, associated to the molecular data discussed above, demonstrate that the shift of the channel from the W state to the R state is not directly induced by the binding of Gβγ as generally proposed, but rather, by the displacement of β from the channel as a consequence of Gβγ binding. Here also, an allosteric model of regulation in which both β3 and Gβγ would remain associated to the channel is difficult to envision. It would seem as an extraordinary coincidence that the biophysical properties of Ca2.1 with β3 and Gβγ bound onto it would be similar to those of Ca2.1 alone, particularly considering that β3 and Gβγ produce such drastic effects alone. With these results, we generated a model of P/Q channel regulation by G-protein (Fig. 5d). In this model, the channel is in the W state in the absence of Gβγ. Activation of G proteins leads to Gβγ binding onto the channel and the departure of the Ca2+ channel β-subunit, resulting in the R state. It should be emphasized that the R state remains a state in which the β-independent G protein regulation remains effective (reduced current amplitude in our study). It should be differentiated from another R state (R‘) in which the channel is neither associated to the Gβγ nor to the β-subunit.

Physiological Importance. Free β-subunit has recently been shown to regulate gene silencing through nuclear relocalization (30). Here, displacement of β from Caβ may represent one of the mechanisms whereby G proteins would trigger the relocalization of β in the nucleus. Hence, this unique mechanism of β dissociation could take part in a new pathway of transcription regulation by G protein-coupled receptors.

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