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Alternative Splicing of the Ca^{2+} Channel β4 Subunit Confers Specificity for Gabapentin Inhibition of $Ca_v2.1$ Trafficking

Patrice M. Mich and William A. Horne

Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado (P.M.M.); Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York (W.A.H.)

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b) Corresponding Author:

William A. Horne
T8 016A, Box 34, Department of Clinical Sciences
College of Veterinary Medicine
Cornell University
Ithaca, New York 14850
Phone: 607-253-4145
Fax: 607-253-3531
e-mail: wah27@cornell.edu

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d) Abbreviations: GBP, gabapentin (1-(aminomethyl)cyclohexaneacetic acid); TEA-OH, tetraethylammonium hydroxide; Ca_v, voltage-gated Ca^{2+} channel

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Abstract

Gabapentin is well-established as an effective treatment for neuropathic pain; however, little is known about its mechanism of action. It binds with high affinity to Ca^{2+} channel α2δ subunits that are expressed in dorsal root ganglia. Mutation of a single α2δ amino acid, R217A, eliminates both gabapentin binding and analgesic efficacy. Gabapentin does not appear to have direct Ca^{2+} channel blocking properties, but does affect overall levels of Ca^{2+} channel surface expression in some circumstances. In this report we examined gabapentin effects on trafficking and voltage-dependent gating properties of recombinant $Ca_v2.1$ Ca^{2+} channel complexes transiently expressed in *Xenopus* oocytes. We also determined electrophysiologically whether gabapentin causes displacement of β subunits from $Ca_v2.1$ complexes. Our principal findings are: 1) gabapentin inhibits trafficking of recombinant $Ca_v2.1$ Ca^{2+} channels in *Xenopus* oocytes; 2) gabapentin inhibition occurs in the presence of the Ca^{2+} channel β4a subunit, but not in the presence of β4b; 3) gabapentin does not affect $Ca_v2.1$ voltage-dependent gating parameters; 4) inhibition of $Ca_v2.1$trafficking is highly dependent on β subunit concentration; and 5) gabapentin inhibition of $Ca_v2.1$ trafficking can be reversed by the α2δ R217A mutation. Overall, our results suggest that gabapentin reduces the number of β4a-bound $Ca_v2.1$ complexes that are successfully trafficked to the plasma membrane. This mechanism may help to explain why gabapentin is both effective and selective in the treatment of neuropathic pain states that involve upregulation of α2δ subunits.

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Gabapentin is well-established as an effective treatment for neuropathic pain (Dworkin et al., 2007). There is also considerable evidence that supports its use in the treatment of a variety of perioperative conditions (preoperative anxiety, post operative nausea and vomiting, hemodynamic response to intubation) and post-surgical acute and chronic pain (Gilron, 2007; Kong and Irwin, 2007). Gabapentin's mechanism of action remains unclear, but has been attributed to effects on several receptors and ion channels. These include activation of $GABA_B$ receptors and K_{ATP} channels, and inhibition of AMPA receptors and voltage-gated Ca^{2+} channels (Cheng and Chiou, 2006). Among the proposed mechanisms, current evidence suggests that modulation of voltage-gated Ca^{2+} channels may be responsible for the analgesic properties of gabapentin and its congener, pregabalin. These compounds bind with high affinity to Ca^{2+} channel $\alpha 2\delta$ subunits (Wang et al., 1999; Dooley et al., 2006) that are expressed in dorsal root ganglia, and that are upregulated considerably under experimental conditions of neuropathic pain (Li et al., 2004). The strongest evidence to date that the $\alpha 2\delta$-1 subunit is the key target for analgesia comes from genetic studies. Knock-in replacement of the wild-type $\alpha 2\delta$-1 subunit with a mutant ($\alpha 2\delta$-1 R217A) incapable of binding pregabalin resulted in complete loss of the drug's analgesic efficacy (Field et al., 2006).

How and to what extent gabapentin and pregabalin modulate Ca^{2+} channels is a matter of much debate. Studies in cultured dorsal root ganglia suggest that gabapentin inhibits a mixed population of Ca^{2+} channel subtypes (Sutton et al., 2002), and that inhibition is dependent on culture conditions and the presence of a Ca^{2+} channel β subunit (Martin et al., 2002). The latter study also showed that inhibitory effects of gabapentin

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were eliminated by pre-treatment with pertussis toxin, invoking a mechanism involving a G protein. These results raise the question as to whether gabapentin's effect on Ca^{2+} channels is direct or indirect. Studies in brain slices showing that gabapentin inhibits K^+-evoked neurotransmitter release have implied that the drug has direct action on $Ca_v2.1$ (P/Q) and $Ca_v2.2$ (N) Ca^{2+} channels (Dooley et al., 2007); however, studies with recombinant expression systems have not supported this conclusion (Davies et al., 2007). These disparate results may be due to channel subunit heterogeneity within different cell-types, variation of gabapentin application and exposure time, and the pathologic state of the tissue examined (hyperalgesic vs. normal). This latter point is emphasized by a report showing that Ca^{2+} currents in mice overexpressing the α2δ–1 subunit are inhibited by gabapentin while currents from wildtype mice are not (Li et al., 2006).

Since the data in support of an acute, external effect of gabapentin on expressed recombinant Ca^{2+} channel function are lacking, in this report we focused on whether gabapentin works inside the cell to affect longer-term processes such as channel assembly and trafficking. The attractiveness of this hypothesis lies in the fact that a major function of α2δ subunits, working jointly with β subunits, is to direct trafficking of Ca^{2+} channel α1 subunits from the endoplasmic reticulum to the plasma membrane (Jarvis and Zamponi, 2007). Surface expression of $Ca_v2.1$ channels increases 7-fold when α2δ-1 is combined in *Xenopus* oocytes with α1A:β4 subunit complexes (Gurnett et al., 1996); β4 subunit addition to α1A: α2δ-1 subunit complexes has similar effects (Helton and Horne, 2002).

Given the observation by Martin et al. that the efficacy of gabapentin is β subunit dependent, we also addressed the question of whether gabapentin efficacy was dependent

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on β4 subunit subtype and concentration, as it is now clear that β subunit effects on surface expression and voltage-dependent gating are separate concentration-dependent processes (Canti et al., 2001; Vendel et al., 2006b). To determine whether gabapentin efficacy was β subunit splice variant dependent, we used two β4 subunit variants (β4a and β4b) that are differentially expressed throughout the nervous system (Helton and Horne, 2002). The β4a form is more widely expressed and is the only β4 splice variant expressed in spinal cord. Moreover, the β4a splice variant is largely expressed at synapses, while β4b is found in cell bodies of neurons and glial cells (Vendel et al., 2006b). In addition, we addressed the question of whether gabapentin exerts its effects on Ca^{2+} channels through regulating α1-β subunit interactions. This is a mechanism of Ca^{2+} channel regulation that has only recently been discovered. Modulation of Ca^{2+} channel gating has been shown to occur, for example, by displacement of β subunits by G proteins (Sandoz et al., 2004). In this report, we used electrophysiological techniques to distinguish between β-bound and β-unbound $Ca_v2.1$ complexes.

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Materials and Methods

Materials. Gabapentin [l-(aminomethyl) cyclohexaneacetic acid] was purchased from Spectrum (New Brunswick, NJ). All other standard chemicals were purchased from Sigma (St. Louis, MO). Leibovitz's L-15 cell culture medium was purchased from Invitrogen (Carlsbad, CA), and collaganase A was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Autoclaved, 0.2 μm-filtered nucelease-free water was purchased from Ambion (Austin, TX).

$Ca_v2.1$ Ca^{2+} Channel Expression in *Xenopus laevis* Oocytes. Complementary RNAs (cRNAs) for each calcium channel subunit (rabbit α1A (BI–2) , rabbit α2δ–1 and α2δ–1_{R217A}, human β4a and β4b) were synthesized in vitro with either T3 or T7 using the mMessage Machine RNA transcription kit (Ambion). Standard methods were used to harvest and prepare *Xenopus laevis* oocytes for cRNA injections. Briefly, oocytes were treated with collagenase A (1.3 mg/ml) in OR2 buffer (in mM: 82.5 NaCl, 2.5 KCl, 1 NaH_2PO_4, 1 $MgCl_2$, 15 HEPES, pH 7.5) for 60 - 90 min to remove the follicular layer. Stage V-VI defolliculated oocytes were sorted and stored primarily in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.5). In some cases, oocytes were stored in OR3 medium (6.85 g/l of Leibovitz's L-15 cell culture medium, 10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 5 mM HEPES, pH 7.5) for several days prior to cRNA injection. Ca^{2+} channel α1, α2δ–1, and either β4a or β4b cRNAs in nuclease-free H_2O were injected into oocytes at various molar ratios (α1:α2δ–1: β ratios of 1:1:0, 1:1:0.3, 1:1:1, 1:1:3, 1:1:10). Unless otherwise

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indicated, these ratios resulted in the following amounts of cRNA per oocyte: α1, 5.6 ng; α2δ–1, 2.4 ng; and either β4a or β4b at 0 ng, 0.45 ng, 1.5 ng, 4.5 ng, or 15 ng. The total volume injected into each oocyte was 46 nl. Injected oocytes were stored at 16°C.

Electrophysiology. Calcium channel currents were recorded 1-6 days post injection by standard two-electrode voltage clamp (Warner OC-725C amplifier, Warner Instrument Corp., Hamden, CT). Microelectrodes were filled with 3M KCl and the resistances of the current and voltage electrodes were 0.5-4.0 MΩ. Data were filtered at 2kHz and sampled at 10kHz. Currents were recorded in a chloride-free bath containing in mM: 5 $Ba(OH)_2$, 5 HEPES, 85 TEA-OH, and 2 KOH, pH adjusted to 7.4 with methanesulfonic acid. Currents used to generate the data in this study ranged from 0.45-2.2μA. In a typical experiment following a 10-20 delay, current levels were measured at 0.5 – 1 hr in one or two oocytes until current levels reached 0.5 – 1.5 μA. Then single oocytes were subjected to both voltage-dependent activation and closed-state inactivation protocols. We have shown previously that following an initial delay, current expression for Ca^{2+} channel complexes containing β subunits is linear up to 2 μA (Helton and Horne, 2002). The activation protocol measured peak barium currents elicited by incremental depolarizing steps of 5mV for 300 msec each from a holding potential of - 80mV (test potentials - 40 to +40 mV). The inactivation protocol measured peak barium currents elicited by 300 msec test depolarization to -5, 0, or 5 mV after a 20 sec conditioning prepulse to voltages between -80 and +30 mV in 10mV incremental steps. Leak currents were between 10 and 150 nA. Only recordings with minimal tail currents were used.

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Data were collected and analyzed using pCLAMP 10 software (Molecular Devices, Sunnyvale, CA) and Microsoft Office Excel 2003 (Microsoft Corp., Redmond WA).

Gabapentin Experiments. Four methods of gabapentin application were studied: acute and chronic external application, direct oocyte injection, and combined direct injection and chronic external application. For acute studies, cRNA injected oocytes were treated with 100 μM gabapentin for 10 minutes prior to and during recording. For chronic studies, cRNA injected oocytes were exposed to 100 μM gabapentin from the moment of cRNA injection to the time of recording (minimum of 20 hours). For injection studies, oocytes were injected with cRNAs and either 1 mM gabapentin (100 μM gabapentin final concentration) or, as control, an equivalent volume of nuclease-free H_2O. For combined injection and chronic exposure studies, oocytes were injected with 10 and 50 mM gabapentin (1 and 5 mM final concntrations), and chronically exposed to 1 and 5 mM gabapentin, respectively. Acute and chronic gabapentin application studies were conducted using α1:α2δ-1:β molar ratios of 1:1:1 and 1:1:10 for both β4a and β4b splice variants. Injected gabapentin studies also included 1:1:0.3 injected oocytes. For gabapentin injection studies, cRNA amounts were: α1, 3.7ng; α2δ–1, 1.6ng; and either β4a or β4b, 0.3, 1.0 or 10 ng (1/3 less cRNA was injected due to additional volume of gabapentin or water). These amounts corresponded to α1, α2δ–1, β4 molar ratios of 1:1:0.3, 1:1:1 and 1:1:10, respectively. For experiments shown in Fig. 5, the total amount of 1:1:1 β4a RNA injected was doubled in order to enhance the rate of current expression.

For α2δ–1 mutant studies, a point mutation (R217A) was inserted into α2δ–1 cDNA using the QuickChange site-directed mutagenesis technique (Stratagene, LaJolla, CA). This mutation eliminates gabapentin binding to the α2δ–1 subunit (Wang et al 1999). The mutation was confirmed by DNA sequencing. $Ca_v2.1$ Ca^{2+} channel α1, α2δ–1(R217A), and β4a cRNA's were injected at a molar ratio of 1:1:1 as described above either with gabapentin or with an equal volume nuclease-free H_2O as control.

Statistical Analysis. Statistical differences were determined using the Mann-Whitney non-parametric test and/or the student's two-sample equal variance t test (Minitab Software, State College, PA). Data are presented as mean +/- SEM.

Results

Concentration-Dependent Effects of β4 Splice Variants on $Ca_v2.1$ Current Expression and Gating. In preparation for gabapentin studies, we first examined the concentration-dependent effects of β4a and β4b splice variants (Helton and Horne, 2002) on the expression and voltage-dependent gating of $Ca_v2.1$ Ca^{2+} channels. Previous studies have shown that regulation of Ca^{2+} channel expression and gating are separable, concentration-dependent functions of β3 and β4a subunits (Canti et al., 2001; Vendel et al., 2006b), but this has not yet been shown for β4b subunits. We therefore studied the effects of increasing concentrations of both β4 splice variants on $Ca_v2.1$ rate of current expression, voltage-dependence of activation and inactivation, and open-state inactivation using α1A:α2δ–1:β4 cRNA molar ratios of 1:1:0, 1:1:0.3, 1:1:1, 1:1:3 and 1:1:10 (Fig.

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1A, 1B insets). As shown in Figs. 1A and 1B, all concentrations of either β4a or β4b enhanced the rate of $Ca_v2.1$ expression relative to α1: α2δ–1 complexes alone. However, in the absence of a β subunit, $Ca_v2.1$ currents did not reach 0.5 μA until > 100 hr, even when using twice the amount of α1:α2δ RNA (2X, Fig. 1 inset). We conclude from these results that in the case of both β4 splice variants, $Ca_v2.1$ expression rate is highly dependent on β subunit concentration.

Figure 2 shows that $Ca_v2.1$ voltage-dependent gating is also highly dependent on the concentration of either β4a or β4b. Expressing β4a and β4b at α1A:α2δ–1:β4 cRNA molar ratios ≥ 1:1:3 had maximal effects on voltage-dependence of activation and inactivation (Fig. 2A-D, Table 1). For both splice variants, current-voltage and inactivation curves shifted leftward to more hyperpolarized potentials by ~15 mV and ~20 mV, respectively. This indicates that, at saturating concentrations of β4 subunit, association with $Ca_v2.1$ α1A subunits makes it easier for the channel to open (requiring less depolarization), but at the same time, decreases the number of Ca^{2+} channels *available* to open. This is a well-recognized phenomenon of β subunits that has yet to be fully understood in the physiological context of neurotransmitter release. Expressing β4a and β4b at α1:α2δ–1:β4 cRNA molar ratios of 1:1:1 had near maximal effects on activation and inactivation, while ratios of 1:1:0.3 caused hyperpolarizing shifts of activation and inactivation intermediate between ratios of 1:1:1 and 1:1:0. We interpret this to mean, as did Canti et al., that currents measured following injection of α1:α2δ–1:β cRNA molar ratios of 1:1:0.3 represent a mixture of current from β-bound and β-unbound α1:α2δ–1 channel complexes. This highlights the separable roles that β subunits play in enhancing Ca^{2+} channel expression and regulating Ca^{2+} channel gating.

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That is, β subunits aid in trafficking α1:α2δ–1 channel complexes to the plasma membrane, but at lower molar ratios don't necessarily remain associated. Fig. 2 E and F and Table 1, show that increasing either β4a or β4b subunit concentration slows open-state inactivation such that the current remaining after 300 msec (R300, % of normalized peak current) increases from ~ 30 to 50%. Taken together, the data in Figs. 1 and 2 indicate that if gabapentin regulation of $Ca_v2.1$ Ca^{2+} channels came about by displacement of either the β4a or β4b subunit during the process of assembly, this would be detected as a slowing of current expression rate, increase in rate of open-state inactivation, and depolarizing shift in the voltage dependence of activation and inactivation.

Gabapentin Inhibition of $Ca_v2.1$ Expression is Dependent on Method of Application and β4 Subunit Concentration and Isoform. Results to this point suggest that $Ca_v2.1$ Ca^{2+} channel expression and gating may be dynamically regulated by the number of β4 subunits available to bind to α1A subunits. We reasoned that if this were the case, gabapentin effects might be dependent on β subunit concentration. As a first step toward testing this hypothesis, we compared the effects of 100 μM gabapentin on the rate of $Ca_v2.1$ current expression in the presence of either β4a or β4b using α1A:α2δ–1:β4 subunit molar ratios of 1:1:1 and 1:1:10. In addition, since a previous study had suggested that gabapentin effects might be time-dependent (Kang et al., 2002), we examined the effects of gabapentin using two different drug exposure times, acute and chronic. Acute exposure consisted of a 10 min treatment with 100 μM gabapentin just prior to and during electrophysiological recording. For chronic exposure, oocytes were

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bathed in 100 μM gabapentin from the time of mRNA injection until the time of electrophysiological recording. Figure 3 shows that, for α1A:α2δ–1:β4a ratios of 1:1:1 (Fig. 3A) and 1:1:10 (Fig. 3C), there is no significant difference between $Ca_v2.1$ expression time in control oocytes and those exposed to gabapentin acutely (1:1:1, p= 0.42; 1:1:10, p=0.30). Chronic exposure to gabapentin, however, significantly slowed $Ca_v2.1$ expression when α1A:α2δ–1:β4a subunits were expressed in a 1:1:1 ratio (Fig. 3A; p= 0.01), but not when the β subunit concentration was increased to a α1A:α2δ–1:β4a ratio of 1:1:10 (Fig. 3B; p= 0.30). These results provided the first indication that the effects of gabapentin were dependent on β4a subunit concentration and time of exposure. By contrast, there was no difference in $Ca_v1.2$ expression times with acute or chronic gabapentin exposure for β4b complexes expressed at α1A:α2δ–1:β4b ratios of 1:1:1 (Fig 3B: acute, p=0.15; chronic, p= 0.30) or 1:1:10 (Fig.3D: acute, p=0.95; chronic, p=0.70). These results indicate that gabapentin effects on $Ca_v2.1$ current expression are also β4 splice variant isoform-specific. Importantly, analysis of gating parameters (Table 2) revealed that gabapentin had no significant effect on the voltage-dependence of activation and inactivation of $Ca_v2.1$ complexes containing either β4a or β4b, even in the case where β4a slowed the rate of $Ca_v2.1$ current expression. This result argues against the notion that gabapentin acts to displace β subunits, as currents expressed in the presence of gabapentin have β-bound properties.

Experiments in which we applied gabapentin externally to $Ca_v2.1$ Ca^{2+} channel complexes containing either β4a or β4b confirmed the findings from other laboratories showing that the drug does not have direct Ca^{2+} channel blocking effects. The fact that gabapentin affected expression of β4a complexes when applied chronically suggests that

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the drug may be taken up slowly by an oocyte transport system. Such a system (leucine-sensitive, $b^{0,+}$ - like transport system) has recently been shown to transport both gabapentin and pregabalin in *Xenopus* oocytes (Su et al., 2005). To bypass the transport system and to examine the effects of gabapentin on $Ca_v2.1$ complexes with reduced β subunit binding, we performed the next set of experiments by injecting gabapentin into oocytes (100 μM final concentration) at the same time that Ca^{2+} channel cRNAs were injected, and included α1:α2δ:β complexes expressed at a ratio of 1:1:0.3. Since we showed that gabapentin's effects were β subunit concentration-dependent, we were particularly interested to see whether the drug would slow the expression of $Ca_v2.1$ complexes at lower concentrations of β4b. Fig 4A shows that, as was the case for externally treated oocytes, gabapentin had no effect on α1:α2δ:β4a complexes expressed at a ratio of 1:1:10. Gabapentin did, however, significantly slow the current expression rate of α1:α2δ:β4a complexes expressed at ratios of 1:1:1 ($p= 0.02$) and 1:1:0.03 ($p= 0.02$). Fig 4B shows that gabapentin had no effect on $Ca_v2.1$ complexes containing β4b, even at the lowest β subunit concentration (α1:α2δ:β4b ratio of 1:1:0.03). Figures 4C and 4D demonstrate that injected gabapentin had no effect on the voltage-dependence of activation and inactivation of 1:1:1 complexes, respectively. This is consistent with the chronic gabapentin exposure result showing that expressed $Ca_v2.1$ currents have β-bound properties.

We next sought to determine whether the effect of GBP on trafficking of β4a complexes was saturable, as would be expected of a drug-receptor interaction, and whether an effect on β4b complexes could be demonstrated at higher GBP concentrations. To do so, we adjusted the amount of total 1:1:1 RNA injected such that

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both β4a and β4b complexes reached 1.5 μA current by 25-30 hr, and we assessed current expression rate at 1 mM and 5 mM GBP (oocytes were both injected and bathed with GBP). Figure 5 shows that 1 mM and 5 mM GBP were equally and significantly effective in reducing the rate of current expression of β4a complexes (1 mM, $p = 0.006$); 5 mM $p = 0.01$), but did not affect the rate of current expression of β4b complexes. Moreover, the effect of higher GBP concentrations on β4a complexes is similar to that seen with 100 μM GBP (Fig. 4), indicating that, under these conditions (1:1:1 molar ratios of injected α1:α2δ;β4a RNA), the maximal effect of GBP on $Ca_v2.1$ trafficking is to reduce the rate of current expression by ~ 30-50%%.

Gabapentin Inhibition of $Ca_v2.1$ Expression is Reversed by the α2δ-1 R217A Mutation. Our results demonstrate that $Ca_v2.1$ Ca^{2+} channel current expression rate is decreased in the presence of intracellular gabapentin; however, experiments to this point have not linked this effect directly to gabapentin binding to α2δ-1. The lack of gabapentin effect on α1:α2δ:β4b complexes expressed under identical conditions rules out a non-specific, toxic effect on expression; but given the variety of proposed gabapentin targets in the literature, it was important to test whether our observed effects on expression rate were specific to gabapentin binding to α2δ-1. Previous studies have identified R217 in an RRR motif as a key residue motif for gabapentin binding to the α2δ–1 subunit (Wang et al., 1999). Moreover, a R217A mutation eliminates both gabapentin binding and analgesic action (Wang et al., 1999; Field et al., 2006). To determine whether our results could be attributed to gabapentin binding to α2δ-1, we performed the next experiments using the α2δ–1 R217A mutant at a

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α1:α2δ–1 R217A:β4a cRNA molar ratio of 1:1:1 in the presence of intracellular 100 μM gabapentin. Figure 6A shows that that there was no significant difference (p=0.52) in $Ca_v2.1$ expression rate when the α2δ–1 R217A mutant was substituted for the wildtype subunit. Likewise, Figs. 6B and 6C show no significant difference in voltage-dependence of activation and inactivation. Comparing $V_{1/2}$ values of activation and inactivation for $Ca_v2.1$ complexes containing either α2δ–1 R217A or wildtype (Table 2), it can be seen that the R217A mutation itself has no effect on gating parameters. Interestingly, this is similar to recording results from dorsal root ganglion cells isolated from R217A knock-in mice, but different from results in which α2δ–1 R217A was coexpressed with $Ca_v2.2$ and β1b (Fields et al., 2006). Nonetheless, we conclude from these experiments that gabapentin decreases $Ca_v2.1$ currents by slowing $Ca_v2.1$ trafficking to the plasma membrane and that this effect is dependent on gabapentin binding to the α2δ-1 subunit.

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Discussion

Our results reveal several interesting properties of neuronal Ca^{2+} channel β4 subunits that not only help to clarify their roles in Ca^{2+} channel trafficking, but also provide insight into the mechanism of action of the widely prescribed analgesic agent, gabapentin. Our initial experiments revealed that when increasing concentrations of either the β4a or β4b splice variant are injected along with a fixed 1:1 ratio of α1 and α2δ subunits, the rate of appearance of plasma membrane Ca^{2+} current increases dramatically. The largest jump in rate (2-3 fold) occurs when the ratio of either β4 subunit increases from 1:1:0 to 1:1:0.3 (α1:α2δ:β). The expression rates then increase incrementally, but in total only 2-3 fold further, when the β subunit ratio is increased in steps from 0.3 to 10 (see Fig. 1). These data indicate that Ca^{2+} channel surface density can vary widely depending on β subunit concentration, and that the trafficking mechanism responsible for regulating surface expression is saturable.

Analyzing the voltage-dependent gating properties of Ca^{2+} currents expressed at these different β subunit concentrations reveals an important feature of the Ca^{2+} channel trafficking mechanism. As the concentration of β subunit is increased incrementally, the voltage-dependence of activation and inactivation of the expressed channels shifts to more hyperpolarized potentials. This biophysical behavior has been described previously for β3 and β4a subunit regulation of α1B and α1A currents, respectively (Canti et al., 2001; Vendel et al., 2006b) and can be explained as follows: Ca^{2+} currents carried by α1:α2δ channel complexes lacking β subunits activate and inactivate over a range of relatively depolarized potentials (curves to the far right in Fig. 2 A,B,C,D); association of

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β subunits with α1:α2δ complexes causes ~ 15 and 20 mV hyperpolarizing shifts in the voltage-dependence of both activation and inactivation, respectively. Since we are measuring whole cell currents, activation and inactivation at intermediate membrane potentials (see 1:1:0.3 results in Fig. 2) represent a mixture of two populations of channel complexes, those with and those without β subunits. These results support the hypothesis first suggested by Canti et al. 2001, that β subunits participate in two separable concentration-dependent processes that regulate Ca^{2+} channel function: 1) trafficking of α1:α2δ complexes to the surface, and 2) regulation of channel gating properties. An important outcome of this two-part mechanism is the possibility that Ca^{2+} channels can exist at the surface without β subunits. This observation is especially intriguing in light of studies showing that β subunit displacement from α1:α2δ complexes is important for regulation of Ca^{2+} channels by G-proteins (Canti et al., 2001; Sandoz et al., 2004), and that β subunits play cellular roles beyond their interactions with Ca^{2+} channels (Ebert et al., 2007; Hidalgo and Neely, 2007). These data when considered together point to a more dynamic role for β subunits in regulation of Ca^{2+} channel properties through mechanisms both in the cytosol and at the cell surface.

We approached our study of gabapentin's effect on $Ca_v2.1$ Ca^{2+} channels with the separable, concentration-dependent effects of β subunits in mind. In agreement with the results from other investigators (Kang et al., 2002; Davies et al., 2006), we found no acute effect of externally applied gabapentin on Ca^{2+} channel current amplitude or voltage-dependent gating behavior. Chronic external exposure, however, did slow the rate of expression of β4a containing complexes injected at a 1:1:1 ratio (α1:α2δ:β).

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Similar results were obtained for $Ca_v2.1$ α1: α2δ: β4 complexes expressed in tsA-201 cells (Hendrich et al., 2008). Interestingly, the gabapentin effect we observed was overcome by increasing the ratio of β4a subunit to 1:1:10 or by co-injecting β4b instead of β4a. These results highlight several important aspects of gabapentin function in the *Xenopus* oocyte system. First, one explanation for the requirement for chronic exposure to gabapentin is that the drug works inside the cell. Thus, in order for gabapentin to have an effect, it must be transported across the cell membrane. In mammalian cells, gabapentin is transported by the L-type amino acid transporter, LAT1 (Uchino et al, 2002); in *Xenopus* oocytes, gabapentin is transported by the Na^+-independent, leucine-sensitive $b^{0,+}$ transport system (Su et al., 2005). In support of an intracellular mechanism of action, we showed that gabapentin is equally effective when injected into the oocyte. Second, the fact that gabapentin's inhibitory effect on Ca^{2+} channel expression could be reversed by increased concentrations of β4a subunit suggests that the drug competes with β subunits in the process responsible for Ca^{2+} channel trafficking. Third, the competition is specific for β4a subunits, as gabapentin has no effect in the presence of β4b. This is perhaps the most striking finding of the study and may help to explain why gabapentin, though it binds to a α2δ subunit that associates with many types of Ca^{2+} channel α1 subunits, has tissue specific effects. The two alternatively spliced forms of the β4 subunit, first described by Helton and Horne (2002) differ in their N-terminal A domains. Very little is known about the function of these domains; however, the two splice forms have been highly conserved throughout evolution (Ebert et al., 2008) and have markedly different cellular distribution patterns (Vendel et al., 2006b). Interestingly, only the β4a form is expressed in spinal cord (Helton et al., 2002), a finding that may factor into

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gabapentin's analgesic actions. The β4a A domain has a unique protein fold (Vendel et al., 2006a) that serves as a protein-protein interaction domain (Vendel et al., 2006b). Less is known about the structure of the β4b A domain, though it is predicted to be largely disordered (unpublished observations). The main question to be answered is whether the β4aA domain promotes gabapentin binding or simply allows it. Conversely, does the β4b A domain prevent gabapentin binding or fail to promote it. Our results cannot distinguish between these two possibilities.

Taken together, our data support the following working model for β subunit-dependent gabapentin inhibition of $Ca_v2.1$ expression (Fig. 7). The salient feature of the model is that transport of α1:α2δ-1 subunit complexes to the cell surface is a β-subunit dependent process, the rate of which is dependent on β subunit concentration. In addition, β subunits may move along the trafficking pathway uncoupled from α1 and α2δ subunits (Spafford et al., 2006). In the figure, increasing the ratio (and therefore the concentration) of β from 1:1:0.3 to 1:1:1 increases the number of β subunits available to transport α1:α2δ-1 subunits along an unknown tract (represented by an upward moving escalator). With the separable functions of β subunits in mind (transport and gating regulation), at the ratio of 1:1:0.3 there are fewer β subunits available at the surface to bind the α1:α2δ-1 complexes compared to when the β subunit is expressed at a ratio of 1:1:1. Therefore, measured currents represent a mixture of β-bound and β-unbound α1:α2δ-1 complexes. By contrast, at the ratio of 1:1:1, there are ample β subunits available at the surface to bind the α1:α2δ-1 complexes (binding is close to saturation). Since addition of gabapentin does not affect voltage dependent gating parameters

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(meaning that the ratio of β-bound and β-unbound α1:α2δ-1 complexes does not change), the availability of β subunits and their ability to interact with α1:α2δ-1 complexes at the cell surface does not appear to be affected by the presence of the drug. This suggests that gabapentin binding to α2δ-1 interferes with an assembly process downstream of the transport of α1:α2δ-1 complexes. Therefore, the overall number of $Ca_v2.1$ complexes at the surface is reduced, but they are all β-bound.

Recent studies have revealed exciting new roles for β subunits in Ca^{2+} channel trafficking that may influence gabapentin effects on $Ca_v2.1$ expression. Several laboratories have shown that RGK GTPases inhibit binding of β subunits to α1 subunits and, as a result, reduce surface expression of Ca_v complexes (Beguin et al., 2001, Finlin et al., 2003). It is possible to speculate that gabapentin binding to α2δ somehow promotes this process, and that the mechanism is β-subunit concentration and subtype specific. Others have shown that the β subunit SH3 domain interacts with dynamin and thereby downregulates Ca_v surface expression by means of endocytosis (Hidalgo and Neely, 2007). Our observations may also be explained by an acceleration of this mechanism by gabapentin. We will explore these possibilities in our future experiments.

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Footnotes

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Figure Legends

Fig. 1. $Ca_v2.1$ current expression rate as a function of β4 subunit concentration. For this and all subsequent figures containing expression rate data, averaged peak currents ± SEM are plotted (*symbols*) against average time (hr) of recording after mRNA injection. *Lines* represent current levels measured from one or two oocytes at 0.5 to 1 hr increments. A, β4a cRNA injected at α1:α2δ-1:β4a molar ratios of 1:1:0 (n=12), 1:1:0.3 (n=9), 1:1:1 (n=11), 1:1:3 (n=11), and 1:1:10 (n=10). *Inset*, agarose gel showing α1, α2δ-1, β4a cRNA mixes used for oocyte injection. B, β4b cRNA injected at α1:α2δ-1:β4b molar ratios of 1:1:0 (n=12), 1:1:0.3 (n=9), 1:1:1 (n=11), 1:1:3 (n=13), and 1:1:10 (n=10). *Inset*, agarose gel showing α1, α2δ-1, β4b cRNA mixes used for oocyte injection.

Fig.2. Effects of β subunit concentration on activation and inactivation of $Ca_v2.1$ Ca^{2+} channels. A, B, current-voltage (I-V) curves resulting from increasing concentrations of β4a and β4b, respectively. Data points represent averaged, normalized peak currents resulting from 300 msec step depolarizations to the indicated membrane potential from a holding potential of – 80 mV. Values for *n* and $V_{1/2}$ ± SEM are listed in Table 1. C, D, voltage-dependence of inactivation curves resulting from increasing concentrations of β4a and β4b, respectively. Data points represent averaged, normalized peak currents resulting from 300 msec depolarizations to -5, 0, or +5 mV from the range of indicated potentials held for 20 sec. Values for *n* and $V_{1/2}$ ± SEM are listed in Table 1. E, F, averaged 300 msec open-state inactivation traces from experiments plotted in A and B,

respectively. Values for *n* and R300 (current remaining after 300 msec) are listed in Table 1.

Fig. 3. Gabapentin effects on of $Ca_v2.1$ current expression rate are dependent on drug treatment time and β subunit subtype and concentration. Averaged peak currents ± SEM are plotted against average time (hr) of recording after mRNA injection. A, B, expression rates of 1:1:1 molar ratio α1:α2δ-1:β4a and α1:α2δ-1:β4b complexes, respectively, in the absence of GBP (no GBP; β4a, n = 9; β4b, n=11), following 10 min treatment with 100 μM GBP (acute GBP; β4a, n=8; β4b, n=10), and following > 36 hr treatment with 100 mM GBP (chronic GBP; β4a n=10; β4b, n=11). Asterisk (*) indicates significant difference from control ($p < 0.05$). C, D, expression rates of 1:1:10 molar ratio α1:α2δ-1:β4a and α1:α2δ-1:β4b complexes, respectively, in the absence of GBP (no GBP; β4a, n=9; β4b, n=10), following 10 min treatment with 100 μM GBP (acute GBP; β4a n=8; β4b, n=9), and following > 20 hr treatment with 100 mM GBP (chronic GBP; β4a n=11; β4b, n=7).

Fig. 4. Effects of injected gabapentin on $Ca_v2.1$ current expression, activation, and inactivation. A, expression rates of 1:1:0.3, 1:1:1, and 1:1:10 molar ratio α1:α2δ-1:β4a complexes following injection of dH_2O (no GBP, solid lines; 1:1:0.3, n=7; 1:1:1, n=14; 1:1:10, n=15), and following injection of gabapentin (~ 100 μM final concentration) into oocytes at the time of cRNA injection (inj GBP, dashed lines; 1:1:0.3, n=7; 1:1:1, n=20; 1:1:10, n=10). Asterisks (*) indicate significant difference from control ($p < 0.05$). B, expression rates of 1:1:0.3, 1:1:1, and 1:1:10 molar ratio α1:α2δ-1:β4b complexes

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following injection of dH_2O (no GBP, solid lines; 1:1:0.3, n=6; 1:1:1, n=10; 1:1:10, n=14), and following injection of gabapentin (~ 100 μM final concentration) into oocytes at the time of cRNA injection (inj GBP, dashed lines; 1:1:0.3, n=6; 1:1:1, n=10; 1:1:10, n=14). C, D, voltage-dependence of activation and inactivation, respectively, of 1:1:1 molar ratio α1:α2δ-1:β4a complexes following injection of dH_2O (no GBP), and following injection of gabapentin (inj GBP, ~ 100 μM final concentration). For C, data points represent averaged, normalized peak currents as in Fig 1C. For D, data points represent averaged, normalized peak currents as in Fig 2D. Values for *n* and $V_{1/2}$ ± SEM are listed in Table 2.

Fig 5. Effects of 1 mM and 5 mM gabapentin on $Ca_v2.1$ current expression rates. A, Expression rates of 1:1:1 molar ratio α1:α2δ-1:β4a complexes following injection of dH_2O (no GBP, solid line, n=9 at 17 hr and n=6 at 27 hr), and following injection of gabapentin (1 mM final concentration, dotted line, n=7 at 17 hr and n=6 at 27 hr; or 5 mM final concentration, dashed lines, n= 7 at 17 hr and n=7 at 27 hr) into oocytes at the time of cRNA injection. Asterisks (*) indicate significant differences from control ($p < 0.05$)B, Expression rates of 1:1:1 molar ratio α1:α2δ-1:β4b complexes following injection of dH_2O (no GBP, solid line, n=6 at 17 hr and n=7 at 27 hr), and following injection of gabapentin (1 mM final concentration, dotted line, n=6 at 17 hr and n=6 at 27 hr; or 5 mM final concentration, dashed lines, n= 7 at 17 hr and n=7 at 27 hr) into oocytes at the time of cRNA injection.

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Fig. 6. The inhibitory effect of gabapentin on $Ca_v2.1$ current expression rate is reversed by the α2δ-1 R217A mutation. A, expression rates of 1:1:1 molar ratio α1:α2δ-1(R217A):β4a complexes following injection of dH_2O (no GBP, solid line, n=7), and following injection of gabapentin (~ 100 μM final concentration) into oocytes at the time of cRNA injection (inj GBP, dashed lines, n=7). C, D, voltage-dependence of activation and inactivation, respectively, of 1:1:1 molar ratio α1:α2δ-1 (R217A):β4a complexes following injection of dH_2O (no GBP), and following injection of gabapentin (inj GBP, ~ 100 μM final concentration). For C, data points represent averaged, normalized peak currents as in Fig 2C. For D, data points represent averaged, normalized peak currents resulting as in Fig 2D. Values for *n* and $V_{1/2}$ ± SEM are listed in Table 2.

Fig 7. Proposed mechanism of action for gabapentin effects on $Ca_v2.1$ Ca^{2+} channel expression. The model suggests that gabapentin works downstream of a trafficking mechanism in which β subunits play a critical role (see Discussion).

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Table 1. Voltage-dependent gating parameters ($V_{1/2}$ ± SEM) for activation and inactivation of $Ca_v2.1$ Ca^{2+} channels resulting from increasing concentrations of either β4a or β4b splice variant. The β subunit concentrations are expressed as molar ratios relative to α1 and α2δ subunits (α1:α2δ:β). R300 is the percentage of current remaining relative to peak values at the end of a 300 msec stimulus.

	n	$V_{1/2}$ activation (mV)	R300 (%)	n	$V_{1/2}$ inactivation (mV)
α1α2δ	12	1.9 ± 0.8	30.4 ± 2.0	10	-18.1 ± 2.6
+ β4a					
1:1:0.3	8	- 4.5 ± 0.6	33.4 ± 1.4	8	- 27.8 ± 2.3
1:1:1	9	- 8.8 ± 1.0	34.4 ± 3.2	8	- 36.3 ± 0.6
1:1:3	11	-13.5 ± 0.7	45.9 ± 1.4	10	- 41.7 ± 0.7
1:1:10	10	-15.8 ± 0.7	55.4 ± 2.2	9	- 42.0 ± 0.3
+ β4b					
1:1:0.3	9	- 6.0 ± 0.7	28.9 ± 2.4	8	- 28.2 ± 0.9
1:1:1	11	-11.8 ± 0.8	48.8 ± 2.6	10	- 38.3 ± 0.5
1:1:3	13	-15.0 ± 0.4	52.6 ± 3.2	12	- 42.6 ± 0.8
1:1:10	10	-14.9 ± 0.6	55.0 ± 3.1	9	- 43.6 ± 0.8

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Table 2. Gabapentin (GBP) effects on voltage-dependent gating parameters ($V_{1/2}$ ± SEM) for activation and inactivation of $Ca_v2.1$ Ca^{2+} channels. Two different concentrations of either β4a or β4b splice variant were examined. The β subunit concentrations are expressed as molar ratios relative to α1 and α2δ subunits (α1:α2δ:β). R300 is the percentage of current remaining relative to peak values at the end of a 300 msec stimulus.

	n	$V_{1/2}$ activation (mV)	R300 (%)	n	$V_{1/2}$ inactivation (mV)
β4a					
1:1:1 no	9	- 8.8 ± 1.0	34.4 ± 3.2	8	- 36.3 ± 0.6
1:1:1 ac	8	-10.4 ± 0.7	40.0 ± 2.1	8	- 39.2 ± 1.3
1:1:1 chr	10	-10.3 ± 0.7	35.9 ± 2.4	10	- 32.3 ± 0.9
1:1:1 inj	14	- 7.2 ± 0.6	45.1 ± 2.0	14	- 30.4 ± 0.6
1:1:1$_{R217A}$	7	- 9.5 ± 0.9	40.3 ± 2.0	7	- 33.8 ± 1.1
1:1:10 no	9	-15.8 ± 0.7	55.4 ± 2.2	9	- 42.1 ± 0.3
1:1:10 ac	8	-15.0 ± 1.2	55.3 ± 4.2	6	- 42.3 ± 0.1
1:1:10 chr	11	-12.5 ± 0.9	34.9 ± 2.4	10	- 40.7 ± 0.4
β4b					
1:1:1 no	11	-11.8 ± 0.8	48.8 ± 2.6	10	- 38.3 ± 0.5
1:1:1 ac	10	-13.2 ± 1.7	40.2 ± 3.2	9	- 38.4 ± 1.2
1:1:1 chr	11	-8.3 ± 0.6	34.5 ± 5.7	10	- 34.4 ± 0.6
1:1:10 no	10	-14.9 ± 0.6	55.0 ± 3.1	10	- 43.6 ± 0.8
1:1:10 ac	9	-14.1 ± 0.4	59.8 ± 3.9	8	- 43.8 ± 0.3
1:1:10 chr	7	-15.2 ± 0.6	54.9 ± 5.8	6	- 43.4 ± 0.4

no, (-) GBP; *ac*, acute (10 min) external treatment with GBP; *ch*, chronic (>20 hr) external treatment with GBP; *inj*, GBP injected into oocytes along with cRNA mixes; *R217A*, $Ca_v2.1$ Ca^{2+} channel complex includes R217A α2δ-1 mutant.

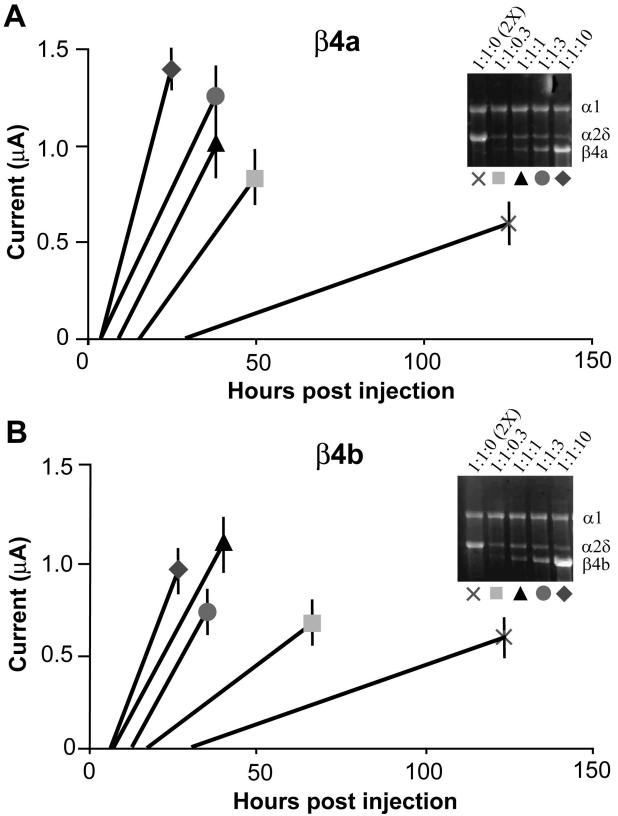
A
β4a
1:1:0 (2X)
1:1:0.3
1:1:1
1:1:3
1:1:10
α1
α2δ
β4a
Current (μA)
1.5
1.0
0.5
0
0
50
100
150
Hours post injection
B
β4b
1:1:0 (2X)
1:1:0.3
1:1:1
1:1:3
1:1:10
α1
α2δ
β4b
Current (μA)
1.5
1.0
0.5
0
0
50
100
150
Hours post injection

Figure 1

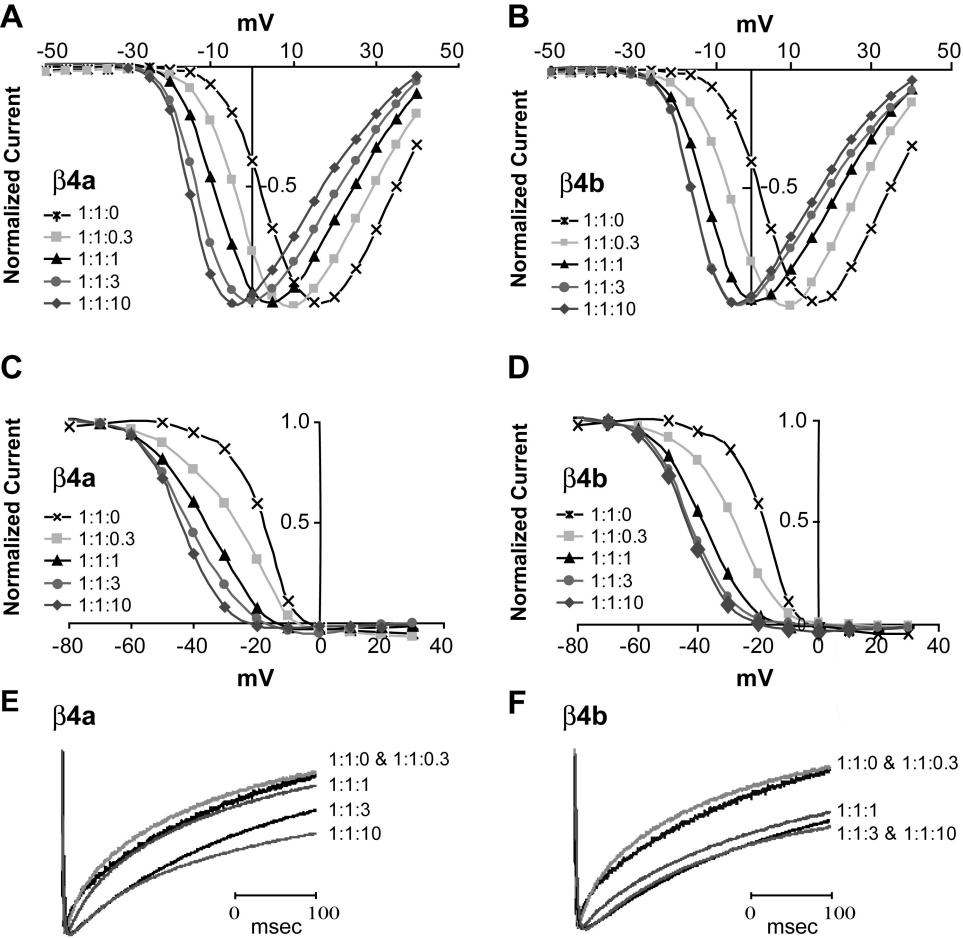
A
mV
-50
-30
-10
10
30
50
Normalized Current
-0.5
β4a
1:1:0
1:1:0.3
1:1:1
1:1:3
1:1:10
B
mV
-50
-30
-10
10
30
50
Normalized Current
-0.5
β4b
1:1:0
1:1:0.3
1:1:1
1:1:3
1:1:10
C
Normalized Current
1.0
0.5
β4a
1:1:0
1:1:0.3
1:1:1
1:1:3
1:1:10
-80
-60
-40
-20
0
20
40
mV
D
Normalized Current
1.0
0.5
β4b
1:1:0
1:1:0.3
1:1:1
1:1:3
1:1:10
-80
-60
-40
-20
0
20
40
mV
E
β4a
1:1:0 & 1:1:0.3
1:1:1
1:1:3
1:1:10
0
100
msec
F
β4b
1:1:0 & 1:1:0.3
1:1:1
1:1:3 & 1:1:10
0
100
msec

Figure 2

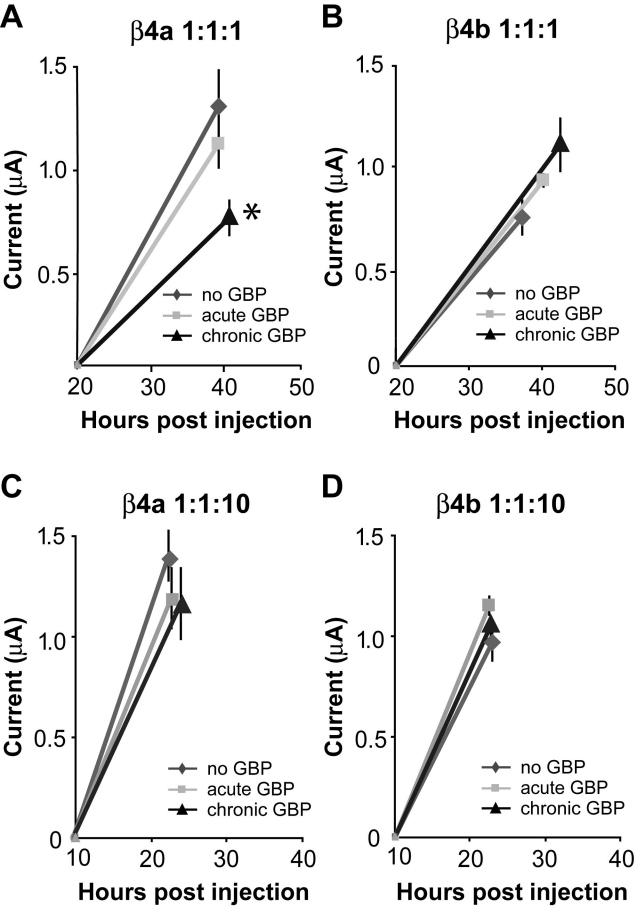
A
β4a 1:1:1
Current (μA)
1.5
1.0
0.5
*
no GBP
acute GBP
chronic GBP
20
30
40
50
Hours post injection
B
β4b 1:1:1
Current (μA)
1.5
1.0
0.5
0
no GBP
acute GBP
chronic GBP
20
30
40
50
Hours post injection
C
β4a 1:1:10
Current (μA)
1.5
1.0
0.5
0
no GBP
acute GBP
chronic GBP
10
20
30
40
Hours post injection
D
β4b 1:1:10
Current (μA)
1.5
1.0
0.5
0
no GBP
acute GBP
chronic GBP
10
20
30
40
Hours post injection

Figure 3

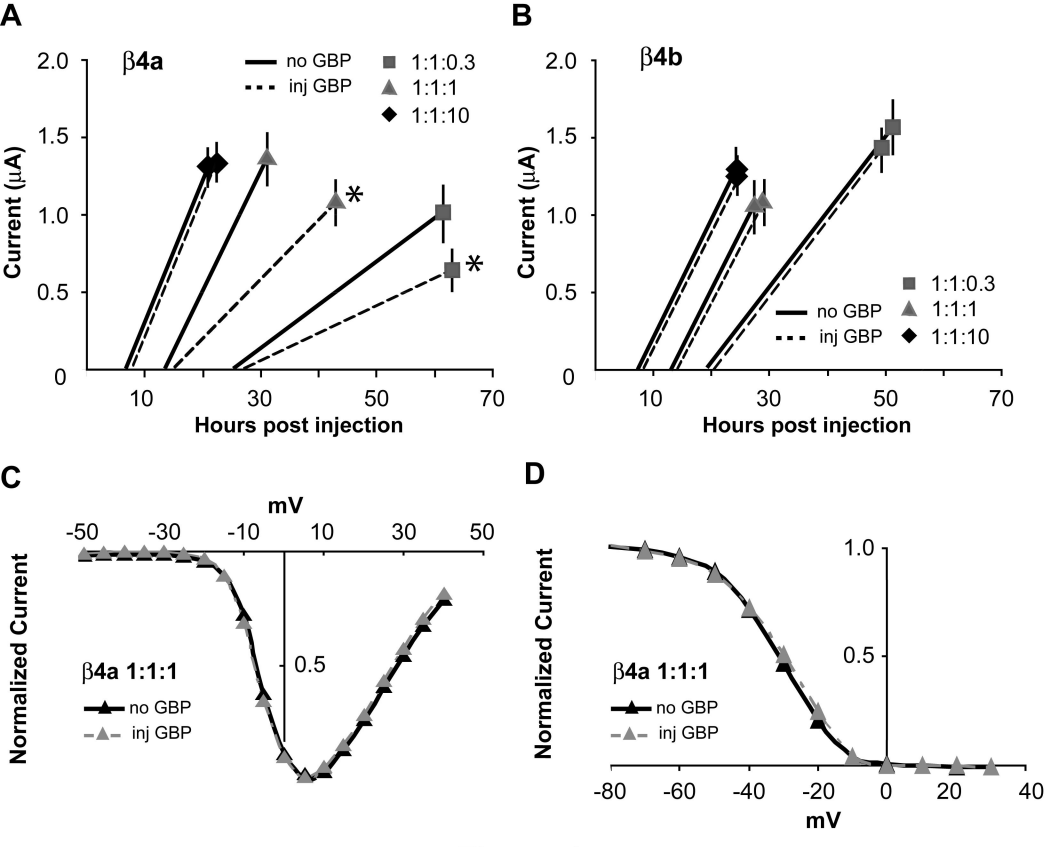
A
β4a
no GBP
inj GBP
1:1:0.3
1:1:1
1:1:10
Current (μA)
Hours post injection
B
β4b
C
mV
β4a 1:1:1
Normalized Current
D
mV

Figure 4

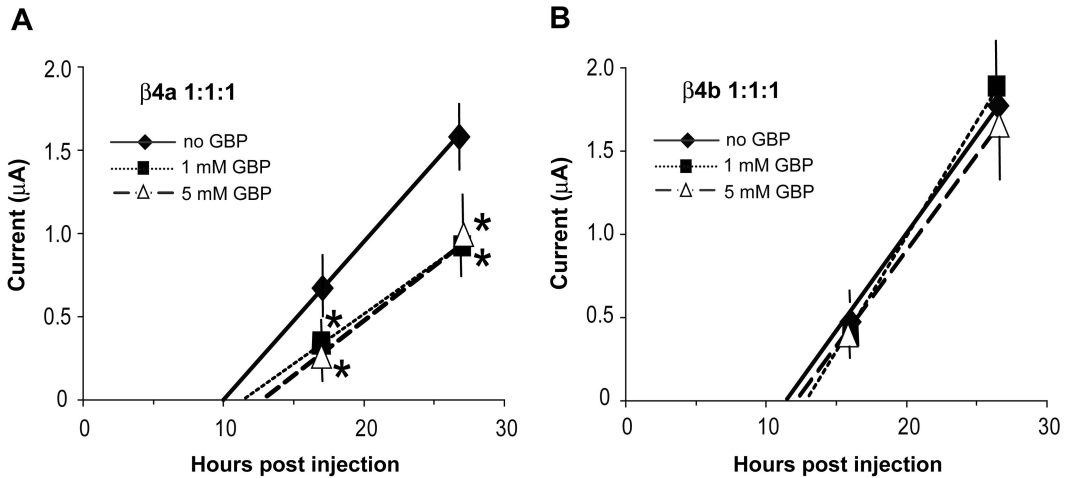
A
β4a 1:1:1
no GBP
1 mM GBP
5 mM GBP
Current (μA)
2.0
1.5
1.0
0.5
0
0
10
20
30
Hours post injection
*
*
*
B
β4b 1:1:1
no GBP
1 mM GBP
5 mM GBP
Current (μA)
2.0
1.5
1.0
0.5
0
0
10
20
30
Hours post injection

Figure 5

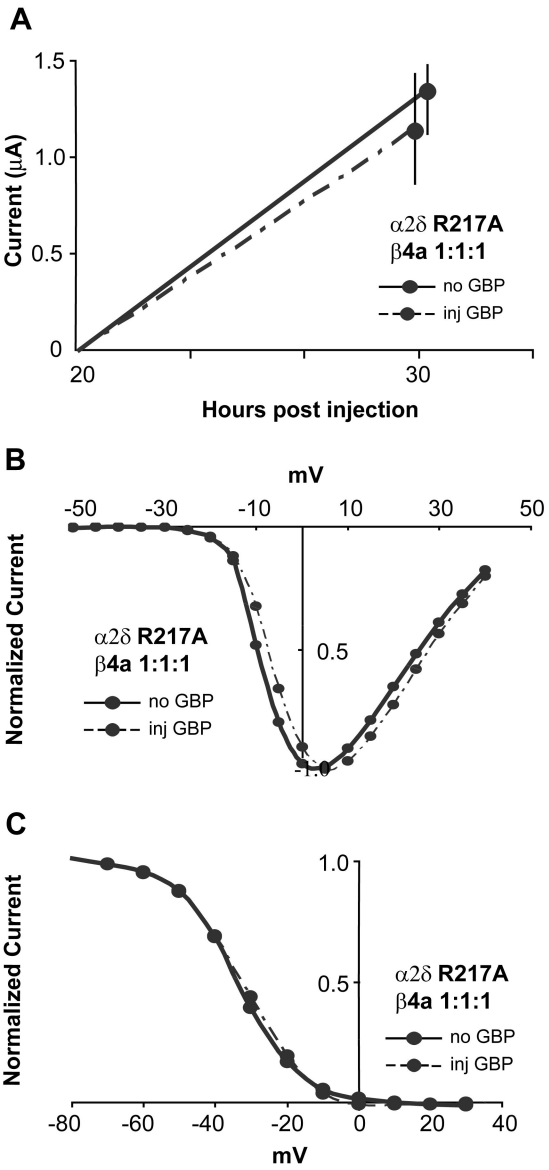
A
1.5
1.0
0.5
0
Current (μA)
20
30
Hours post injection
α2δ R217A
β4a 1:1:1
no GBP
inj GBP
B
mV
-50
-30
-10
10
30
50
Normalized Current
0.5
α2δ R217A
β4a 1:1:1
no GBP
inj GBP
C
1.0
0.5
Normalized Current
-80
-60
-40
-20
0
20
40
mV
α2δ R217A
β4a 1:1:1
no GBP
inj GBP

Figure 6

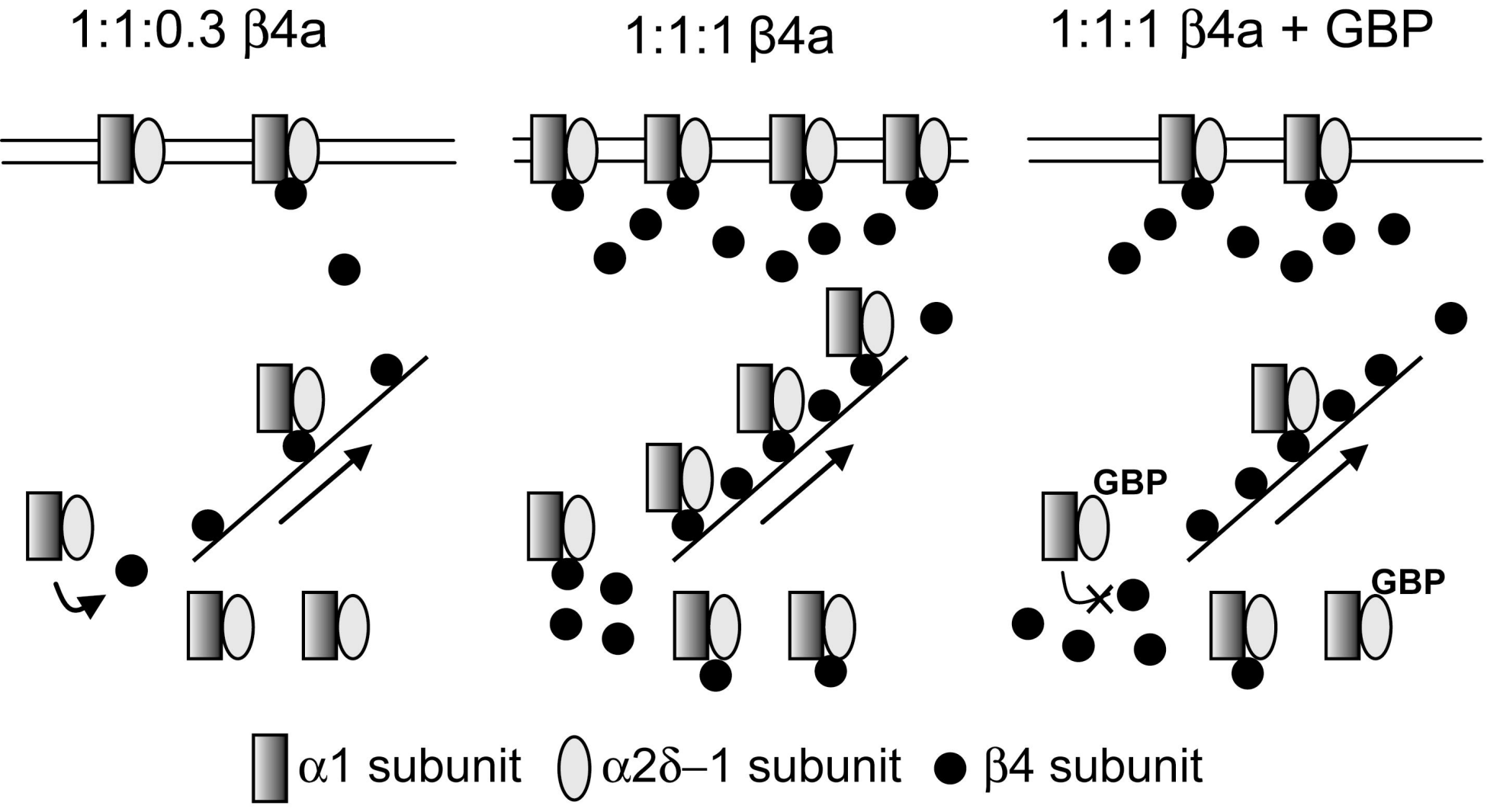
1:1:0.3 β4a
1:1:1 β4a
1:1:1 β4a + GBP
GBP
GBP
α1 subunit
α2δ–1 subunit
β4 subunit

Figure 7