Pharmacological disruption of calcium channel trafficking by the $\alpha_2\delta$ ligand gabapentin

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Edited by Richard W. Tsien, Stanford University School of Medicine, Stanford, CA, and approved January 15, 2008 (received for review September 20, 2007)

The mechanism of action of the antiepileptic and antinociceptive drugs of the gabapentinoid family has remained poorly understood. Gabapentin (GBP) binds to an exofacial epitope of the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 auxiliary subunits of voltage-gated calcium channels, but acute inhibition of calcium currents by GBP is either very minor or absent. We formulated the hypothesis that GBP impairs the ability of $\alpha_2\delta$ subunits to enhance voltage-gated Ca^{2+}channel plasma membrane density by means of an effect on trafficking. Our results conclusively demonstrate that GBP inhibits calcium currents, mimicking a lack of $\alpha_2\delta$ only when applied chronically, but not acutely, both in heterologous expression systems and in dorsal root-ganglion neurons. GBP acts primarily at an intracellular location, requiring uptake, because the effect of chronically applied GBP is blocked by an inhibitor of the system-L neutral amino acid transporters and enhanced by coexpression of a transporter. However, it is mediated by $\alpha_2\delta$ subunits, being prevented by mutations in either $\alpha_2\delta$-1 or $\alpha_2\delta$-2 that abolish GBP binding, and is not observed for $\alpha_2\delta$-3, which does not bind GBP. Furthermore, the trafficking of $\alpha_2\delta$-2 and Ca_V2 channels is disrupted both by GBP and by the mutation in $\alpha_2\delta$-2, which prevents GBP binding, and we find that GBP reduces cell-surface expression of $\alpha_2\delta$-2 and $Ca_V2.1$ subunits. Our evidence indicates that GBP may act chronically by displacing an endogenous ligand that is normally a positive modulator of $\alpha_2\delta$ subunit function, thereby impairing the trafficking function of the $\alpha_2\delta$ subunits to which it binds.

Voltage-gated Ca^{2+}channels (VGCCs) are heteromeric complexes. The Ca_V1 and Ca_V2 subfamilies are made up of a pore-forming $\alpha1$ subunit, associated with a membrane-anchored, predominantly extracellular, $\alpha_2\delta$ subunit (for review see ref. 1) and an intracellular β subunit (for review see ref. 2). Mammalian genes encoding four $\alpha_2\delta$ subunits have been identified (for reviews see refs. 2 and 3). The topology of the $\alpha_2\delta$ protein was first determined for $\alpha_2\delta$-1 and is thought to generalize to all $\alpha_2\delta$ subunits (for reviews see refs. 1 and 4). They are type I transmembrane proteins, the exofacial α_2 subunit being disulfide-bonded to a transmembrane δ subunit, formed by posttranslational cleavage of the $\alpha_2\delta$ preprotein (5).

The mechanism of action of the antiepileptic and antinociceptive drugs of the gabapentinoid family has remained poorly understood. Gabapentin (GBP) itself was originally developed as an analog of γ-amino-butyric acid (GABA), but is now believed to have no effect on GABA receptors or transporters (for review see ref. 6). The first key to understanding the mechanism of action of GBP came from purification of the GBP-binding protein from porcine brain (7), which was identified as the $\alpha_2\delta$-1 auxiliary subunit of VGCCs. It is now known that GBP binds to an exofacial epitope present in both the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits (for reviews see refs. 1 and 8). However, although it was originally reported that GBP application results in acute inhibition of calcium currents (9, 10), in most studies, acute inhibition by GBP is either very minor or absent (for review see ref. 1). Furthermore, electrophysiological studies of synaptic transmission are also equivocal, with some studies reporting inhibition by GBP (11), and other studies reporting no inhibition (12). When neurotransmitter release is measured, there appears to be little or no effect on depolarization-stimulated release of several different neurotransmitters, but inhibition of release when this has been enhanced by specific mediators (13–15). In general, none of these studies points to the mechanism of action being a simple inhibition of calcium currents.

We formulated the hypothesis that GBP impairs the ability of $\alpha_2\delta$ subunits to enhance VGCC plasma membrane density, via an effect on trafficking. This would be in agreement with the finding that *in vivo* responses to gabapentinoid drugs are fairly slow in onset (16), and GBP does not inhibit acute pain (for reviews see refs. 6 and 17).

Results

Inhibition of $Ca_V2.1$ and $Ca_V2.2$ Currents by Chronic, but Not Acute, GBP. We first compared the ability of GBP to inhibit calcium currents, either acutely, or when included for 40 h in the culture medium after transfection of tsA-201 cells with $Ca_V2.1/\beta4$ and $\alpha_2\delta$-2. Chronic incubation with GBP (1 mM) reduced currents formed with the WT $\alpha_2\delta$-2 by 72.2 ± 4.2% at +15 mV (Fig. 1*A*). Furthermore, whereas the coexpression of $\alpha_2\delta$ subunits typically shifted the voltage dependence of steady-state inactivation to more negative potentials (Fig. 1*B*), this was depolarized by 9.3 mV in the continued presence of GBP (Fig. 1*B*). Thus, chronic GBP mimicked a lack of influence of $\alpha_2\delta$ on the currents. In confirmation, a similar effect of chronic GBP was observed when $Ca_V2.1/\beta4$ was transfected into a cell line stably expressing $\alpha_2\delta$-2, where peak currents were 53.4 ± 14.0% smaller when cells were cultured with 1 mM GBP [supporting information (SI) Fig. 5*A*]. Furthermore, the steady-state inactivation was again depolarized by 11 mV by GBP (SI Fig. 5*B*), and the currents showed significantly slower inactivation (SI Fig. 5 *C* and *D*). In contrast, when GBP was applied either acutely for 10 min (Fig. 1*C*), or for 3–6 h before recording (n = 8, data not shown), it had no effect on I_{Ba} in the same system.

A high concentration of GBP was used initially, because our hypothesis required GBP either to be taken up by system-L transporters and subsequently to bind intracellular $\alpha_2\delta$ subunits and affect forward trafficking or, alternatively, to bind directly to cell surface $\alpha_2\delta$ subunits and affect trafficking at the level of endocytosis or recycling. For both of these sites, it will be

Author contributions: J.H. and A.T.V.M. contributed equally to this work; J.H., A.T.V.M., and A.C.D. designed research; J.H., A.T.V.M., F.H., J.W., and A.C.D. performed research; J.H., A.T.V.M., M.N.-R., K.W., J.S., and A.D. contributed new reagents/analytic tools; F.H. and A.C.D. analyzed data; and A.C.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0708930105/DC1.

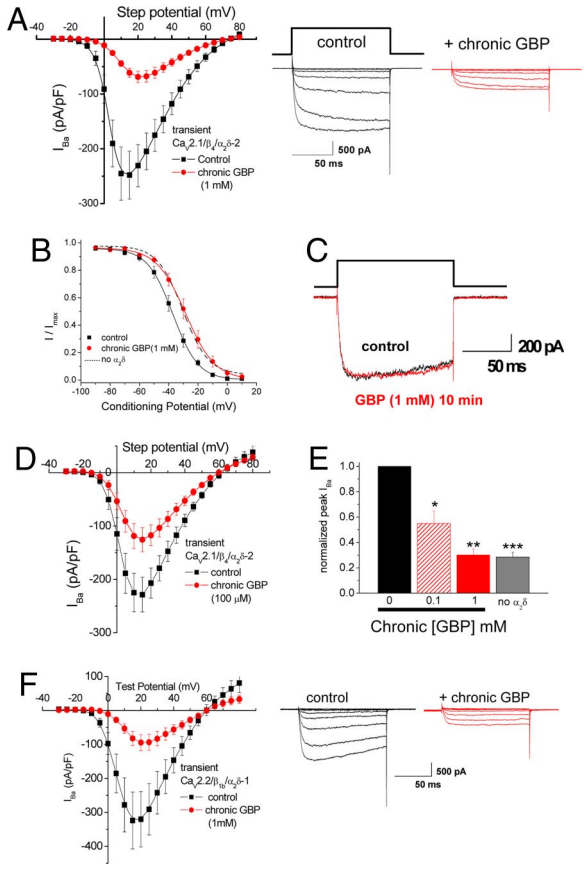

Fig. 1. GBP is effective to inhibit I_{Ba} after heterologous expression, when applied chronically but not acutely. (*A*) (*Left*) Current density–voltage (IV) relationships for Ca$_V$2.1/β4/α$_2$δ-2 currents in the absence or presence of chronic GBP (1 mM, red circles, $n = 11$) for ≈40 h (or H_2O as control, black squares, $n = 13$) from immediately after transfection of tsA-201 cells until recordings were performed. The reduction in peak I_{Ba} at +15 mV was statistically significant ($P = 0.0013$, Student's two-tailed t test). (*Right*) Examples of currents resulting from step potentials from −90 mV to between −15 and +15 mV in 5-mV increments, under control conditions and in the presence of GBP. Calibration bars refer to both sets of traces. (*B*) Steady-state inactivation data were obtained from the cells treated as described in *A*. Chronic GBP (1 mM, red circles, $n = 12$) and control (black circles, $n = 13$). The steady-state inactivation in the absence of α$_2$δ subunits is provided for comparison (dashed line, $n = 11$). Data are fit to a single Boltzmann equation. The $V_{50,inact}$ was 37.6 ± 2.1 mV in the presence of α$_2$δ-2, −30.8 ± 4.4 mV in the absence of α$_2$δ (dashed line), and −28.3 ± 2.7 mV in the continued presence of GBP ($P = 0.0103$ compared with the absence of GBP, Student's two-tailed t test). (*C*) GBP (1 mM) applied acutely for 10 min had no effect on Ca$_V$2.1/β4/α$_2$δ-2 currents. Examples of currents resulting from step potentials from −90 mV to +10 mV, under control conditions and after application of GBP for 10 min, to a cell whose initial current amplitude had stabilized. Currents are representative of $n = 5$ cells, where the peak current (red trace) after application of GBP was 99.0 ± 7.1% of its initial value, before GBP application (black trace). (*D*) IV relationships for Ca$_V$2.1/β4/α$_2$δ-2 currents from cells cultured in the absence or presence of 100 μM GBP applied chronically as described in *A*. GBP (red circles, $n = 10$) and control (black squares, $n = 14$). The reduction in peak I_{Ba} at +15 mV was statistically significant ($P = 0.0215$, Student's two-tailed t test). (*E*) The percentage of inhibition of peak current density by GBP (100 μM, hatched red bar, $n = 10$; and 1 mM, solid red bar, $n = 11$) was determined for each experiment and compared with the reduction observed in the absence of α$_2$δ (gray bar, $n = 14$). The statistical significance of the reduction was determined relative to the data in the presence of α$_2$δ-2 and absence of GBP for each individual set of experiments; *, $P = 0.0013$; **, $P = 0.021$; ***, $P = 0.00004$, Student's two-tailed t test. (*F*) (*Left*) IV relationships for Ca$_V$2.2/β1b/α$_2$δ-1 currents in the absence or presence of chronic GBP (1 mM, red circles, $n = 10$) for ≈40 h or the equivalent amount of H_2O as control (black squares, $n = 8$). (*Right*) Examples of currents resulting from step potentials from −90 mV to between −15 and +15 mV in 5-mV increments, under control conditions (black traces) and in the presence of GBP (red traces). Calibration bars refer to both sets of traces.

competing with other large neutral amino acids (18, 19), and both isoleucine and leucine are present at 800 μM and valine at 400 μM in the culture medium. However, plasma concentrations of 70–120 μM GBP are clinically relevant (20), and chronic application of 100 μM GBP also produced a significant reduction of I_{Ba}, by 45.0 ± 9.9% (Fig. 1 *D* and *E*). It also slowed the inactivation of the currents, again a hallmark of calcium channel currents not influenced by α$_2$δ (SI Fig. 6 *A* and *B*). In the prolonged presence of GBP, the Ca$_V$2.1/β4/α$_2$δ-2 current density approached that observed in the absence of α$_2$δ subunits (Fig. 1*E*).

The effect of chronic GBP was also observed in the presence of α$_2$δ-1, because 1 mM GBP reduced peak Ca$_V$2.2/β1b/α$_2$δ-1 currents by 70.5 ± 7.3% (Fig. 1*F*), and depolarized the midpoint for steady-state inactivation from −55.5 ± 3.4 mV to −39.7 ± 2.6 mV ($n = 4$ for both, data not shown). These results also provide evidence that GBP is not selective for a particular subtype of Ca$_V$2 channel composition.

Prevention of the Effect of Chronic GBP by Mutation of α$_2$δ-1 or α$_2$δ-2. Mutation of a single amino acid, R217A, in an RR*R* motif in α$_2$δ-1 (21, 22), or the equivalent residue R282A in α$_2$δ-2 (23), has been found almost completely to abrogate GBP binding. To demonstrate that the effect of chronic GBP was indeed due to binding to α$_2$δ subunits, we examined whether it would have any effect on currents formed by Ca$_V$2.1/β4 cotransfected with R282A-α$_2$δ-2. Chronic incubation with GBP (1 mM) did not inhibit these currents (Fig. 2*A*) or result in any shift in the steady-state inactivation (Fig. 2*B*). Furthermore, there was no effect of GBP on currents formed with Ca$_V$2.2/β1b/R217A-α$_2$δ-1 (Fig. 2*C*), or Ca$_V$2.1/β4/α$_2$δ-3 (peak I_{Ba} was −286.58 ± 32.3 pA/pF at +15 mV for control, $n = 8$; compared with −258.7 ± 46.8 pA/pF for chronic GBP, $n = 9$). These results agree with the fact that none of these α$_2$δ subunits bind GBP (data not shown).

Evidence for an Intracellular Site of Action of GBP. The *in vivo* potency of GBP has been shown to depend both on binding to α$_2$δ subunits and on substrate activity for system-L amino acid transporters (24, 25), attributed to the requirement for the zwitterionic drug to pass the blood–brain barrier (18). To determine whether the effect of chronic GBP also required its uptake at the single-cell level (as outlined in Fig. 2*D*), we included the inhibitor 2-(−)-endoamino-bicycloheptene-2-carboxylic acid (BCH) in the medium, either alone or together with GBP for 40 h. BCH has a very low affinity for displacement of GBP binding to α$_2$δ-1 and α$_2$δ-2 subunits (26), and it had no effect on I_{Ba} amplitude when applied alone at 10 mM. The peak current density at +15 mV was −330.2 ± 84.7 pA/pF ($n = 7$) for control and −333.0 ± 73.2 pA/pF ($n = 8$) in the presence of BCH. However, chronic BCH prevented the effect of chronic GBP both to reduce the expressed I_{Ba} (Fig. 2*E*) and to depolarize its steady-state inactivation (data not shown). This effect of BCH is likely to result from its ability to block GBP uptake (18).

To obtain further evidence that GBP uptake is required for its effect, we used *Xenopus* oocytes, which express only a low level of endogenous system-L transporter activity (27). We found that chronic incubation with 200 μM GBP only significantly inhibited Ca$_V$2.2/β1b/α$_2$δ-2 currents (by 57%, Fig. 2*F*) when a system-L transporter protein LAT4 (27) was coexpressed. Expression of LAT4 had no significant effect on the peak I_{Ba} at +5 mV, which was −0.49 ± 0.12 μA ($n = 34$) and −0.41 ± 0.06 ($n = 41$) μA in the absence and presence of LAT4, respectively, from four separate experiments.

We also found that chronic incubation of oocytes for 40 h in the presence of L-leucine (400 μM) significantly enhanced I_{Ba} only when LAT4 was coexpressed (Fig. 2*G*). Furthermore, L-leucine did not enhance the small currents obtained in the absence of α$_2$δ, despite the presence of LAT4 (Fig. 2*G*). This is

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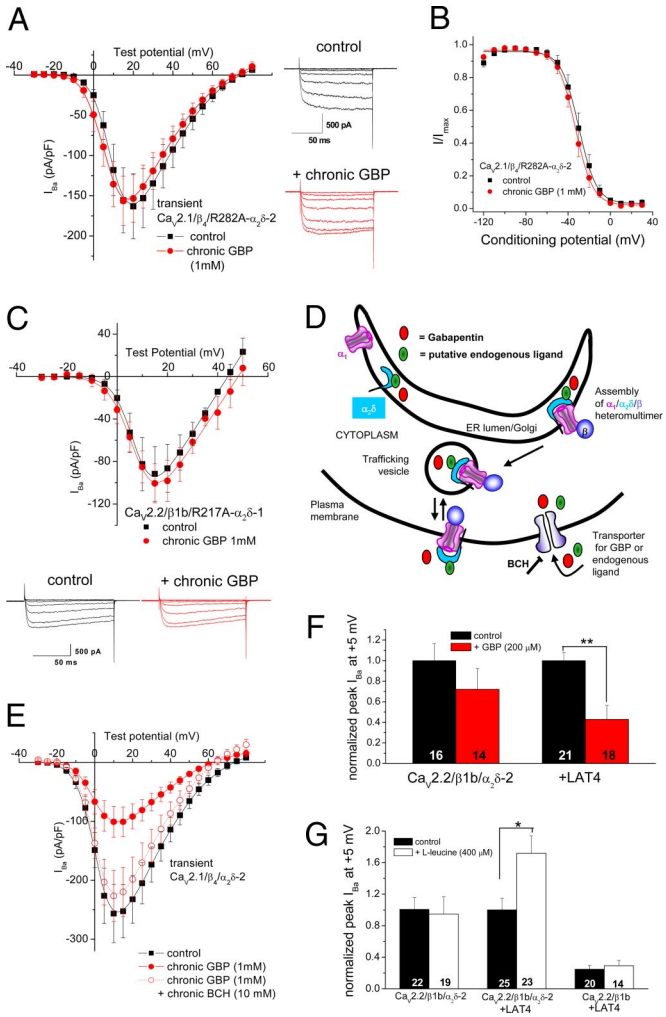

Fig. 2. The effect of GBP requires both binding to the $\alpha_2\delta$ subunit and uptake by neutral amino acid transporter. (*A*) (*Left*) IV relationships for Ca$_V$2.1/β4/R282-$\alpha_2\delta$-2 currents in the absence or presence of chronic GBP (1 mM, red circles, $n = 12$) for ≈40 h or the equivalent amount of H_2O as control (black squares, $n = 12$), from immediately after transfection until recordings were performed. (*Right*) Examples of currents resulting from step potentials from −90 mV to between −15 and +15 mV in 5-mV increments under control conditions and in the presence of 1 mM GBP. Calibration bars refer to both sets of traces. (*B*) Steady-state inactivation data were obtained from the same cells as described in *A*. Chronic GBP (1 mM, red circles, $n = 5$) and control (black squares, $n = 7$). (*C*) (*Upper*) IV relationships for Ca$_V$2.2/β1b/R217A-$\alpha_2\delta$-1 currents in the absence or presence of chronic GBP. The cDNAs were transfected into tsA-201 cells that were then incubated with GBP (1 mM, red circles, $n = 5$) for ≈40 h or the equivalent amount of H_2O as control (black squares, $n = 4$), from immediately after transfection until recordings were performed. (*Lower*) Examples of currents resulting from step potentials from −90 mV to between −15 and +15 mV in 5-mV increments under control conditions and in the presence of 1 mM GBP. Calibration bars refer to both sets of traces. (*D*) Diagram illustrating some of the various sites at which GBP (red circles) or the putative endogenous ligand (green circles) may act on $\alpha_2\delta$ subunits. The effect of GBP may be to displace an endogenous ligand and impair the ability of $\alpha_2\delta$-1 and $\alpha_2\delta$-2 to increase VGCC concentration at the plasma membrane. Thus, GBP may exert its effect on intracellular $\alpha_2\delta$ subunits during maturation and trafficking of the VGCC complex to the plasma membrane and/or bind to cell surface $\alpha_2\delta$ subunits and affect recycling from the plasma membrane. BCH is a competitive inhibitor of system-L transport. (*E*) IV relationships for Ca$_V$2.1/β4/$\alpha_2\delta$-2 currents either under control conditions (black squares, $n = 8$) or after chronic treatment with GBP (1 mM), in the absence (red filled circles, $n = 7$) or presence (red open circles, $n = 10$) of the inhibitor of system-L transport, BCH (10 mM) applied chronically from 1 h before GBP. The reduction in peak I_{Ba} by GBP alone at +10 mV was statistically significant compared with control ($P = 0.0464$, Student's two-tailed *t* test). (*F*) I_{Ba} was measured in *Xenopus* oocytes after injection of Ca$_V$2.2/β1b/$\alpha_2\delta$-2 cDNAs, together with either a control cDNA (nonconducting Kir2.1-AAA (30) (*Left*) or with mLAT4 (27) (*Right*). Oocytes were incubated without (black bars) or with 200 μM GBP (red bars) from 1 h after the time of injection until recording between 40 and 48 h later. To combine data from several experiments, the mean peak control I_{Ba} at +5 mV was normalized and the effect of GBP determined relative to control. The numbers of determinations are shown on the bars. Statistical significance: **, $P = 0.0042$ for the effect of GBP in the mLAT4 condition only (two-way ANOVA and Bonferroni's post hoc test). (*G*) As in *E*, with the combinations of transfected subunits indicated below the bars. Oocytes were incubated without (black bars) or with (white bars) 400 μM L-leucine. The statistical significance is $P = 0.011$ (*) for the effect of L-leucine in the mLAT4 condition and only in the presence of $\alpha_2\delta$-2.

compatible with the view that an endogenous low-molecular-weight ligand, such as L-leucine itself, may normally occupy the GBP-binding sites on $\alpha_2\delta$-1 and $\alpha_2\delta$-2 and be a positive modulator required for the full functionality of $\alpha_2\delta$ subunits (1, 23). The existence of an endogenous ligand was previously suggested by the observation that the apparent affinity for GBP is increased by 5- to 10-fold upon dialysis or partial purification of $\alpha_2\delta$ subunits (19, 23).

In agreement with this hypothesis, for both R217A-$\alpha_2\delta$-1 and R282A-$\alpha_2\delta$-2, the peak currents were consistently smaller, compared with the WT $\alpha_2\delta$ subunits, by ≈62.5% and 34.5% respectively (see also refs. 23 and 28). However, they were significantly greater than in the absence of any $\alpha_2\delta$ subunit, where I_{Ba} at +15 mV was -33.9 ± 5.8 pA/pF for Ca$_V$2.2/β1b ($n = 9, P < 0.05$) and -65.1 ± 8.8 pA/pF for Ca$_V$2.1/β4 ($n = 13$, from ref. 24). This is compatible with the hypothesis that the RRA mutant $\alpha_2\delta$ subunits are defective either in forward trafficking or in maintaining mature VGCC complexes at the plasma membrane.

Chronic GBP Reduces Plasma Membrane Expression of Calcium Channels. To probe further whether GBP affected VGCC trafficking, we then examined the subcellular distribution of VGCC subunits, using Ca$_V$2.1 with an exofacial double hemagglutinin (HA) tag (Ca$_V$2.1–2HA, Fig. 3 *A–C*). Under control conditions, Ca$_V$2.1 and $\alpha_2\delta$-2 were colocalized, both intracellularly (Fig. 3*A*) and also at the plasma membrane, as seen most clearly in nonpermeabilized cells (Fig. 3*B*). The plasma membrane expression in nonpermeabilized cells was quantified from low-magnification images including those in Fig. 3*C*, in which cell-surface expression is seen across the entire cell because of the depth of the optical section (4.5 μm for these images) and the flattened nature of the cells. Chronic incubation with 100 μM and 1 mM GBP significantly reduced the expression of both Ca$_V$2.1 and $\alpha_2\delta$-2 at the plasma membrane (Fig. 3*C*) as well as increasing the nonuniform intracellular clustering of both subunits (Fig. 3*A*). A very similar result was observed for GFP–Ca$_V$2.2, which is expressed throughout transfected Cos-7 cells, typically showing a fairly uniform distribution [SI Fig. 7 (29)]. Using permeabilized cells, we found that WT $\alpha_2\delta$-2 colocalizes with GFP-Ca$_V$2.2, both intracellularly and at the plasma membrane (SI Fig. 7*Ai*). Chronic GBP application resulted in regions of intracellular clustering in most cells, both for $\alpha_2\delta$-2 and for GFP-Ca$_V$2.2 (SI Fig. 7*A ii* and *iii*, quantified in SI Fig. 7*B*).

To quantify further the effect of GBP on cell-surface expression of $\alpha_2\delta$-2, we used cell-surface biotinylation and found that chronic incubation with GBP (100 μM-1 mM) reduced the proportion of $\alpha_2\delta$-2 expressed at the cell surface (Fig. 3*D*). The biotinylation procedure did not induce cell permeabilization, and this was not affected by chronic incubation with GBP, because no biotinylation of the intracellular protein Akt was observed (Fig. 3*D*).

In contrast, chronic GBP did not reduce the level of R282A-$\alpha_2\delta$-2 expressed at the cell surface (SI Fig. 8*A*). Furthermore, in the presence of R282A-$\alpha_2\delta$-2, there was a less uniform distribution of both the mutant $\alpha_2\delta$-2 and GFP-Ca$_V$2.2 than that seen

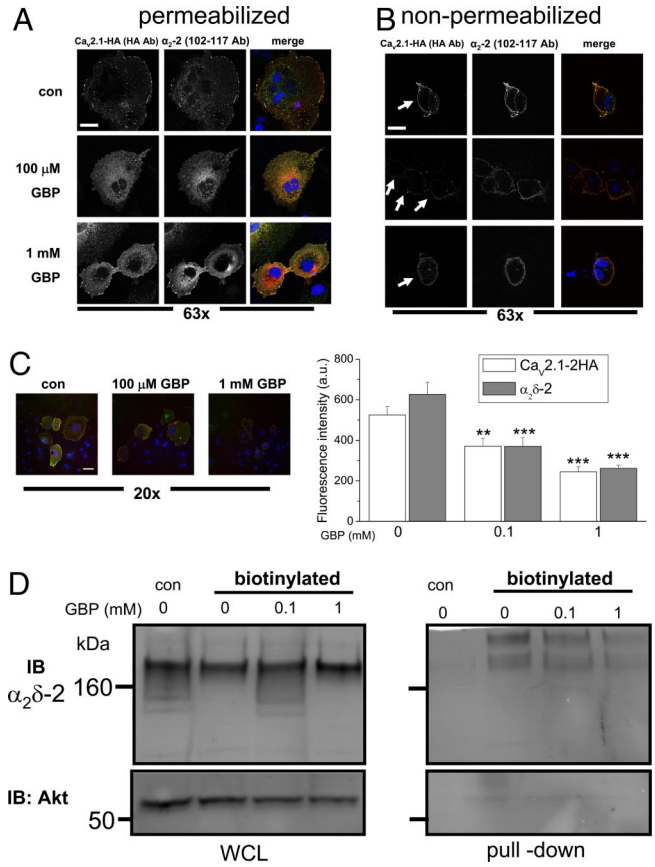

Fig. 3. Effect of chronic GBP on the plasma membrane localization of $Ca_V2.1$ and $\alpha_2\delta$-2 in COS-7 cells. (*A*) $Ca_V2.1$–2HA was cotransfected with $\beta4$ and $\alpha_2\delta$-2 and cultured for 48 h either in the absence or in the presence of GBP (100 μM or 1 mM). Cells were then fixed and permeabilized before immunocytochemical localization of $Ca_V2.1$ (HA Ab, *Left*) and $\alpha_2\delta$-2 [α_2-2 (102–117) Ab, *Center*], by using a magnification ×63 objective. Merged images are shown (*Right*) ($Ca_V2.1$ is shown in green and $\alpha_2\delta$-2 in red, with regions of colocalization in orange–yellow). Nuclear staining (blue, DAPI) is shown in the merged images. Images show 1-μm optical sections of data representative of three independent experiments. (Scale bar: 30 μm.) No signal was observed in nontransfected cells or in the absence of primary Abs (data not shown). (*B*) Images are obtained as in *A*, but cells were not permeabilized. The transfected cell(s) in each image are identified by an arrow. Similar results were obtained in three independent experiments. (Scale bar: 30 μm.) (*C*) (*Left*) Merged images of nonpermeabilized cells taken with a magnification ×20 objective to show a larger field of view for cells transfected as in *A* and cultured for 48 h either in the absence or in the presence of GBP (100 μM or 1 mM). The optical section for these images is 4.5 μm, and, as the cells are flattened, staining is seen over most of the cell surface. (Scale bar: 60 μm.) (*Right*) Quantification of cell-surface fluorescence intensity per cell area for $Ca_V2.1$ (white bars) and $\alpha_2\delta$-2 (gray bars) from magnification ×20 images under control conditions ($n = 22$ cells) and in the presence of 100 μM GBP ($n = 19$ cells) and 1 mM GBP ($n = 24$ cells). **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA, with Bonferroni's post hoc test. (*D*) The effect of GBP (100 μM and 1 mM) was determined on cell-surface expression of $\alpha_2\delta$-2 (*Upper*), with the intracellular protein Akt as control (*Lower*). (*Left*) Whole-cell lysate (WCL). (*Right*) Streptavidin pull-down of biotinylated proteins, immunoblotted with α_2-2 (102–117) or Akt Ab. The leftmost lane is from nonbiotinylated control cells (con). The proportion of $\alpha_2\delta$-2 at the cell surface was estimated from the ratio of the biotinylated $\alpha_2\delta$-2 to the amount of $\alpha_2\delta$-2 in the WCL. Data are from five experiments; 4.93 ± 1.92% of total $\alpha_2\delta$-2 was at the plasma membrane, and this was reduced compared with control by 19.8 ± 7.6% and 30.4 ± 14.4% in the chronic presence of 100 μM and 1 mM GBP, respectively ($n = 5$, $P < 0.05$, repeated-measures ANOVA and Bonferroni's post hoc test).

with WT $\alpha_2\delta$-2, and less uniform colocalization of the R282A-$\alpha_2\delta$ subunit and GFP-$Ca_V2.2$ (SI Fig. 8 *B* and *C*). GBP had no additional effect on the subunit distribution found with R282A-$\alpha_2\delta$-2 (SI Fig. 8 *B* and *C*).

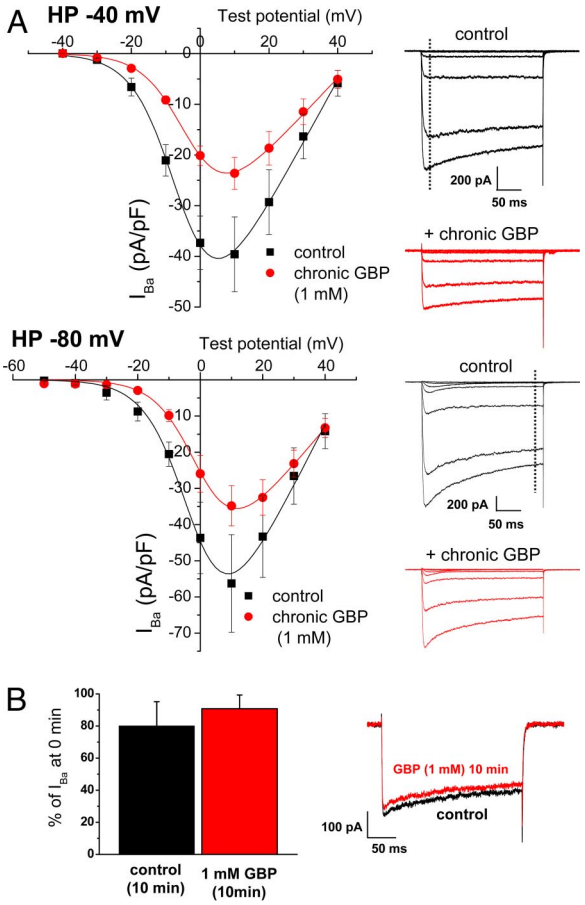

Fig. 4. Effect of GBP on native calcium currents in DRGs. (*A*) (*Upper Left*) IV relationships for HVA I_{Ba} recorded from rat DRGs cultured for 3 days in the absence (black squares, $n = 22$) or presence of GBP (1 mM, red circles, $n = 19$). (*Upper Right*) Example HVA currents from a holding potential (V_H) of −40 mV to between −30 and +10 mV in 10-mV steps. Upper traces (black) control; lower traces (red) chronic GBP (1 mM). I_{Ba} was measured at 20 ms, as indicated by the dotted line. I_{Ba} at 0 mV was significantly inhibited by chronic GBP ($P = 0.02$, Student's two-tailed t test). (*Lower*) As above, except V_H was −80 mV, and I_{Ba} amplitude was measured at the end of the 220-ms step, as indicated by the dotted line. I_{Ba} was obtained in the absence (black squares, $n = 12$), or presence of GBP (1 mM, red circles, $n = 13$). (*B*) Acute application of GBP (1 mM) had no effect on native DRG HVA currents. (*Left*) Bar chart showing lack of effect of perfusion for 10 min of control medium (black bar) or GBP (1 mM, red bar) to cells whose initial current amplitude had stabilized ($n = 6$ for each). (*Right*) Examples of currents resulting from a step potential from V_H −40 mV to +10 mV under control conditions (black trace) and from the same cell after application of GBP (red trace) for 10 min.

Chronic GBP Inhibits Native Calcium Currents in DRG Neurons. To determine the relevance of our findings to native VGCCs in a neuronal system relevant to the therapeutic use of GBP, we examined whether GBP had a similar chronic effect on I_{Ba} density in cultured adult dorsal root ganglion (DRG) neurons. We found a marked reduction in peak high voltage-activated (HVA) calcium currents (by 50.7 ± 11.2% at 0 mV, $n = 19$, $P = 0.02$), when DRGs were incubated with 1 mM GBP for 3 days (Fig. 4*A*). Contamination with T-type current was avoided by measuring peak current from a holding potential of −40 mV, although a similar result was obtained when measuring I_{Ba} at the end of a 50-ms step from −80 mV (Fig. 4*A*). In contrast, acute application of GBP for 10 min had no effect on adult DRG I_{Ba} (Fig. 4*B*).

Discussion

Subsequent to the finding that GBP binds to certain VGCC $\alpha_2\delta$ subunits (19), the evidence is now very strong that gabapentinoid

drugs have their therapeutic effect via this route, because an R217A $\alpha_2\delta$-1 knockin mouse develops neuropathic pain in response to nerve injury, but this is unresponsive to either GBP or its analog pregabalin (28). We now show that GBP is an inhibitor of VGCC trafficking, rather than a direct inhibitor of calcium currents, and thus exerts its inhibitory effects primarily on intracellular $\alpha_2\delta$ subunits. We have shown that $\alpha_2\delta$ subunits have their main effect on VGCC trafficking (30), and that the von Willebrand factor-A (VWA) domain is key to this process. It is notable that GBP binds to the two $\alpha_2\delta$ subunits that have VWA domains with perfect metal-ion adhesion site (MIDAS) motifs (31). It has been postulated that all such VWA domains undergo a conformational change on binding the protein ligand of this domain (30, 31), and one might speculate that gabapentinoid drugs could interfere with this function of the VWA domain. The reduction in cell-surface expression of $\alpha_2\delta$-2 and $Ca_V2.1$ in the presence of GBP and the reduced colocalization of R282A-$\alpha_2\delta$-2 with $Ca_V2.2$ provides supporting evidence for this hypothesis.

The findings presented here could account for the lack of effect, or small responses, reported to acute GBP in different experimental systems, both on calcium currents and on synaptic transmission (for review see ref. 1). Previous studies examining more prolonged exposure to GBP have also shown either no inhibition or a small inhibition of current amplitude (32, 33). Our finding that the effect of GBP requires its uptake into individual cells explains the requirement for a relatively high concentration of GBP, because it will need to outcompete large neutral amino acids for uptake at this site. It is clear that the trafficking of VGCCs and their insertion and removal from the plasma membrane proceeds dynamically (see, for example, ref. 34), and these rates are likely to depend on the physiological state of the neurons, and the amount of $\alpha_2\delta$ subunits present, which both enhance forward trafficking and decrease the turnover of VGCCs (30, 35). Insertion of VGCCs into the plasma membrane of presynaptic terminals or axons may occur rapidly under certain conditions, for example, after the induction of neuropathic pain, when $\alpha_2\delta$-1 subunit levels are elevated. In this regard, it has been reported that GBP slowly inhibited calcium currents over a period of minutes in DRGs from $\alpha_2\delta$-1-overexpressing mice (mimicking the neuropathic pain state), but not WT mice (36).

In conclusion, the action of GBP elucidated here, to inhibit VGCC trafficking and plasma membrane expression, represents a previously uncharacterized mechanism of drug action, and points the way forward for development of screens for drug development.

Methods

Tissue Culture and Heterologous Expression of cDNAs. Details are provided in *SI Methods*.

Electrophysiology. Calcium channel currents were recorded essentially as described (37, 38) and detailed in *SI Methods*.

Immunoblotting. Immunoblot analysis was performed essentially as described (30). SDS/PAGE-resolved samples were transferred to PVDF membranes and probed with relevant primary Abs as described (30) and with the appropriate horseradish peroxidase-conjugated secondary Abs, followed by enhanced chemiluminescence detection.

Immunocytochemistry and Imaging The method used is essentially as described (39) and detailed in *SI Methods*.

Biotinylation Assay At 72 h after transfection with the cDNAs described, cells were rinsed three times with PBS and then incubated with PBS containing 1 mg/ml Sulfo-NHS-SS-Biotin (Pierce) for 30 min at 4°C. The biotin solution was removed and rinsed two times with PBS containing 100 mM glycine (pH 8.0) at room temperature to quench the reaction. The cells were gently rinsed three times with PBS and then lysed. Half of the cell lysate was loaded onto a 3–8% Tris-Acetate gel to determine total protein expression. Equal amounts of biotinylated proteins were precipitated by adding 50 μl of streptavidin–agarose beads (Pierce) and incubated overnight at 4°C. The streptavidin–agarose beads were washed three times and incubated with 100 mM DTT in 2$\times$ Laemmli sample buffer for 1 h at 37°C. Eluted proteins were then resolved by SDS/PAGE. Immunoblotting for the cytosolic protein Akt was used as a control to determine that intracellular proteins were not biotinylated as a result of cellular damage.

Statistical Analysis Data are given as means $\pm$ SEM, and the statistical tests used are either ANOVA with Bonferroni's post hoc test or Student's two-tailed *t* test, as stated.

ACKNOWLEDGMENTS. We thank Dr. S. Bodoy (University of Barclona, Barcelona) for mouse LAT4 cDNA and Kanchan Chaggar (University College London) for tissue culture. This work was supported by Medical Research Council Grant G0700368, the Wellcome Trust, Austrian Science Fund Grant P17109, and the British Council.

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