Gabapentin-mediated inhibition of voltage-activated Ca\(^{2+}\) channel currents in cultured sensory neurones is dependent on culture conditions and channel subunit expression

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Abstract

We have used the whole cell patch clamp method and fura-2 fluorescence imaging to study the actions of gabapentin (1-(aminoethyl) cyclohexane acetic acid) on voltage-activated Ca\(^{2+}\) entry into neonatal cultured dorsal root ganglion (DRG) neurones and differentiated F-11 (embryonic rat DRG×neuroblastoma hybrid) cells. Gabapentin (2.5 \(\mu\)M) in contrast to GABA (10 \(\mu\)M) did not influence resting membrane potential or input resistance. In current clamp mode gabapentin failed to influence the properties of evoked single action potentials but did reduce the duration of action potentials prolonged by Ba\(^{2+}\). Gabapentin attenuated high voltage-activated Ca\(^{2+}\) channel currents in a dose- and voltage- dependent manner in DRG neurones and reduced Ca\(^{2+}\) influx evoked by K\(^{+}\) depolarisation in differentiated F-11 cells loaded with fura-2. The sensitivity of DRG neurones to gabapentin was not changed by the GABA\(_B\) receptor antagonist saclofen but pertussis toxin pre-treatment reduced the inhibitory effects of gabapentin. Experiments following pre-treatment of DRG neurones with a PKA-activator and a PKA-inhibitor implicated change in phosphorylation state as a mechanism, which influenced gabapentin action. Sp- and Rp-analogues of cAMP significantly increased or decreased gabapentin-mediated inhibition of voltage-activated Ca\(^{2+}\) channel currents. Culture conditions used to maintain DRG neurones and passage number of differentiated F-11 cells also influenced the sensitivity of Ca\(^{2+}\) channels to gabapentin. We analysed the Ca\(^{2+}\) channel subunits expressed in populations of DRG neurones and F-11 cells that responded to gabapentin by Quantitative TaqMan PCR. The data obtained from this analysis suggested that the relative abundance of the Ca\(^{2+}\) channel \(\beta_2\) and \(\alpha_2\delta\) subunit expressed was a key determinant of gabapentin sensitivity of both cultured DRG neurones and differentiated F-11 cells. In conclusion, gabapentin inhibited part of the high voltage-activated Ca\(^{2+}\) current in neonatal rat cultured DRG neurones via a mechanism that was independent of GABA receptor activation, but was sensitive to pertussis toxin. Gabapentin responses identified in this study implicated Ca\(^{2+}\) channel \(\beta_2\) subunit type as critically important to drug sensitivity and interactions with \(\alpha_1\) and \(\alpha_2\delta\) subunits may be implicated in antihyperalgesic therapeutic action for this compound.

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1. Introduction

Gabapentin (1-(aminoethyl) cyclohexane acetic acid; Neurontin\textsuperscript{\textregistered}) is an effective anticonvulsant (Taylor et al., 1998), and has recently been shown to also have a number of other therapeutic effects on various forms of pain. These effects of gabapentin may involve direct effects on the excitability of sensory neurones. Gabapentin is structurally similar to the inhibitory amino acid GABA and was initially investigated as a potential GABA mimetic with high lipid solubility for blood–brain barrier penetration (Rogawski and Porter, 1990). However, gabapentin’s anticonvulsant actions appeared not to be
dependent on GABA mimetic effects (Rock et al., 1993) either at the GABA receptor level or through an influence of GABA re-uptake (Taylor et al., 1998). To date the only high affinity binding site identified for gabapentin is the α,δ subunit complex of voltage-activated Ca\(^{2+}\) channels (Gee et al., 1996; Marias et al., 2001) a finding which makes modulation of Ca\(^{2+}\) entry through these channels a potential mechanism of action. Gabapentin has been shown to inhibit voltage-activated Ca\(^{2+}\) channels in a number of neuronal preparations. These include, adult rat brain neurones from the neocortex, striatum and external globus pallidus (Stefani et al., 1998), Ca\(^{2+}\) influx into rat neocortical synaptosomes (Meder and Dooley, 2000) and Ca\(^{2+}\) currents in neonatal rat cultured sensory neurones (Alden and Garcia, 2001). Furthermore gabapentin attenuated evoked release of neurotransmitters including, noradrenaline from rat neocortical slices (Fink et al., 2000) and peptide-enhanced glutamate release from neocortical and trigeminal nucleus slices (Maneuf and McKnight, 2000). However, apparently conflicting data has been obtained from rat freshly dissociated nodose ganglia and dorsal root ganglion (DRG) neurones (Rock et al., 1993) and hippocampal granule neurones from patients with temporal lobe epilepsy (Schumacher et al., 1998) where gabapentin showed no modulation of voltage-activated Ca\(^{2+}\) currents. With respect to potential mechanisms of actions of gabapentin, recent data has raised further questions. Specifically, gabapentin has been shown to inhibit voltage-activated Ca\(^{2+}\) channel subunits. Calcium imaging experiments were also conducted on F-11 cells (embryonic rat DRG×neuroblastoma hybrid) because these cells can support a greater number of consistent Ca\(^{2+}\) transients in response to KCl-induced depolarisation compared with primary cultures of DRG neurones (Sutton et al., 2002). Some of the data in this manuscript has previously been published in abstract form (Martin et al., 2000; McClelland et al., 2000).

2. Methods

2.1. Cell culture

Primary cultures of rat sensory neurones from dorsal root ganglia were used in this study, but two different media were used to maintain the neurones because the culture conditions influenced the sensitivity of neurones to gabapentin. Dorsal root ganglia from decapitated 2-day old Wistar rats were dissected out and the neurones dissociated enzymatically with collagenase (0.125% in PBS for 13 min), trypsin (0.25% in PBS for 6 min) and mechanically by trituration. The sensory neurones were plated on to laminin-polynornithine coated coverslips and bathed in F-14 culture medium (Imperial Laboratories) supplemented with 10% horse serum (Gibco), penicillin (5000 IU/ml), streptomycin (5000 mg/ml), NaHCO\(_3\) (14 mM) and nerve growth factor (NGF-2.5S; 10–20 ng/ml). The cultures were maintained for up to 2 weeks at 37°C in humidified air with 5% CO\(_2\) and were re-fed with fresh culture medium after 5–7 days. In some experiments cultures of DRG neurones were pre-treated for 16 h with 500 ng/ml pertussis toxin (PTX) at 37°C. A second approach to maintain cultured DRG neurones was also used and this method primarily differed from that outlined above in the serum and NGF content of the culture medium. Rat DRG neurones were cultured as described above with the following exceptions. DRGs were initially re-suspended and cultured in DMEM-based media (Gibco+10% FBS, 2 mM L-glutamine, penicillin (5000 IU/ml), streptomycin (5000 mg/ml), NGF-7S (100 ng/ml, Promega) and were treated with cytosine arabinoside (2.5 μM) for the first 48 h in culture (37°C in air containing 5% CO\(_2\)). Cells were then transferred to a defined DMEM-based culture media containing: 1% FCS (Gibco), 2 mM L-glutamine, penicillin (5000 IU/ml), streptomycin (5000 mg/ml), NGF-7S (100 ng/ml, Promega), and 1% N\(_2\) supplement (Gibco). Cells were re-fed with fresh culture media after 2–3 days.

In the present study we initially investigated the actions of gabapentin on cultured sensory neurones to further characterise the effects of gabapentin on electrophysiological properties of DRG neurones. Comparisons with GABA responses have been made to examine the possibility that some of the analgesic properties of gabapentin could be mediated via a GABA receptor mech-
Undifferentiated F-11 cells were obtained from Dr M.C. Fishman (Massachusetts General Hospital, Boston, MA). The F-11 cells were grown as a monolayer on glass coverslips in 30 mm culture dishes and bathed in Ham’s F-12 culture medium. The culture medium was supplemented with 2 mM L-glutamine, 15% Hyclone foetal bovine serum, HAT (100 µM hypoxanthine/400 nM aminopterin/16 µM thymidine), 100 units/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37°C in humidified air with 5% CO₂, and passed twice a week using mechanical cell dissociation. The F-11 cells used in this study were differentiated by placing the cells for 3 days in differentiating medium which was similar to the medium described above except that it was serum free and contained 500 µM dibutryl cyclic AMP and 10 ng/ml NGF-7S. The actions of gabapentin were assessed using differentiated F-11 cells, which had been passaged between 2 and 10 times.

2.2. Electrophysiology

Electrophysiological experiments were conducted at room temperature (18–20°C) using the whole cell variant of the patch clamp technique. Membrane potential, input resistance and the properties of conventional action potentials and action potentials recorded in the presence of Ba²⁺ were studied in current clamp mode. Additionally, voltage-activated Ca²⁺ channel currents carried by either Ca²⁺ or Ba²⁺ were recorded from cultured neonatal rat DRG neurones. The patch pipettes with resistances of 3–9 MΩ were made from Pyrex borosilicate glass tubing (1.4/1.6 mm outer diameter, 0.8/1.0 mm bore with 0.15 mm fibre attached to the inside wall, Plowden and Thompson Ltd, Dial Glass Works) using a two-stage vertical microelectrode puller (David Kopf Instruments, Tujuncn, USA, Model 730). An Axoclamp 2A switching amplifier (Axon Instruments) operated at a switching frequency of 15 kHz was used. Recordings during initial experiments in current clamp mode were made using a patch pipette filling solution containing in millimolar: KCl, 140; EGTA, 5; MgCl₂, 0.1; MgCl₂, 2.0; HEPES, 10.0; ATP, 2.0; and extracellular solution containing in millimolar: NaCl, 130; KCl, 3.0; CaCl₂ or BaCl₂, 2.0; MgCl₂, 0.6; NaHCO₃, 1.0, HEPES 10.0 and glucose 5.0. For recording Ca²⁺ channel currents the patch pipettes were filled with CsCl-based solution containing in millimolar: 140 CsCl, 0.1 CaCl₂, 5 EGTA, 2 MgCl₂, 2 ATP, 10 HEPES. The pH and osmolarity of the patch pipette solutions were corrected to 7.2 and 310–320 mOsm/l with Tris and sucrose. The extracellular bathing solution used contained in mM: 130 choline chloride, 2 CaCl₂ or BaCl₂, 3 KCl, 0.6 MgCl₂, 1 NaHCO₃, 10 HEPES, 5 glucose, 25 tetrathylammonium chloride, 0.0025 tetrodotoxin (Sigma). The pH and osmolarity of this extracellular bathing solution were corrected to 7.4 and 320 mOsm/l with NaOH and sucrose, respectively. The recording solutions used in these experiments were designed to attenuate voltage-activated Na⁺ and K⁺ currents and isolate voltage-activated Ca²⁺ currents. Gabapentin and Bay K8644 were applied to the extracellular environment by low-pressure ejection from a blunt pipette positioned about 50–100 µm away from the cell being recorded. Voltage-activated Ca²⁺ currents were evoked by 100 ms voltage step commands applied every 30 s and records were captured and stored on digital audiotape using a Biologic digital tape recorder (DTR 1200). Analysis of data was performed off-line using Cambridge Electronic Design voltage clamp analysis software (Version 6.0). All voltage-activated Ca²⁺ currents had scaled linear leakage and capacitance currents subtracted to obtain values for the net inward Ca²⁺ current. Data are given as mean±standard error of the mean (s.e.m.) values and statistical significance was determined using a paired or independent Student’s t-test as appropriate.

The sensitivity of HVA calcium channel currents carried by Ba²⁺ to gabapentin was also assessed after 48-h culture utilising the whole-cell mode of the patch clamp technique. Extracellular recording saline comprised of (mM): Choline chloride (130), TTX (0.0025), TEA (25), KCl (3), BaCl₂ (2–5), MgCl₂ (0.6), NaHCO₃ (1), HEPES (10), Glucose (4), pH 7.4 NaOH, 320 mOsm/l (sucrose). Intracellular patch pipettes were filled with (mM): CsCl (140), EGTA (10), CaCl₂ (0.1), MgCl₂ (2.0), HEPES (10), and ATP (2). The osmolarity and pH were adjusted to 310 mOsm/l and pH 7.2 with sucrose and Tris. All experiments were performed at room temperature (20–22°C). Whole cell patch clamp recordings were performed using an Axopatch 200 amplifier (Axon Instruments Inc., Foster City, CA) linked to a personal computer equipped with pCLAMP Version 8.0. Patch pipettes (World Precision Instruments 1B12OF-3) were pulled using a Sutter P-87 microelectrode puller and showed typical resistances of 2–4 MΩ. Series resistance had typical values of 8–12 MΩ and was electronically compensated by at least 90%. Recordings were low-pass filtered at 2 kHz using the built in Bessel filter of the amplifier and digitised at 5 kHz using a Digidata 1200 A/D converter (Axon Instruments). Peak inward currents (Iₒ) were activated from a Vh of −80 mV using 100 ms steps to Vc of 0 mV every 15 s. To control for full equilibration with the internal patch solution data were obtained from currents that had been allowed to stabilise for up to 10 min before any measurements were made. Drugs were applied by gravity-fed bath perfusion from an outlet placed close to the recorded cell. Data were analysed using Clampfit (Axon Instruments, Inc.) All curve fitting was carried out in GraphPad Prism Version 3.0. Unless stated otherwise, data are expressed as mean±s.e.m, numbers in parentheses displayed on the figures reflect numbers of experiments; and P values
given reflect unpaired two tailed Student’s t-tests. Stocks of gabapentin, Sp- and Rp-CAMPS (RBI) were prepared in water and stored at −20°C.

2.3. Calcium imaging

Cultured differentiated F-11 cells were incubated for 1 h in NaCl-based extracellular solution contained (in mM): NaCl, 130; KCl, 3.0; MgCl₂, 0.6; CaCl₂, 2.0; NaHCO₃, 1.0; HEPES, 10.0; glucose, 5.0 and 0.01 fura-2AM (Sigma, 1 mM stock in dimethylformamide). The pH was adjusted with NaOH to 7.4 and the osmolarity to 310–320 mOsm with sucrose. The cells were then washed for 10–20 min with NaCl-based extracellular solution to remove the extracellular fura-2AM and this period allowed cytoplasmic de-esterification of the Ca²⁺ sensitive fluorescent dye. The cells were constantly perfused with NaCl-based extracellular solution (1–2 ml/min) and viewed under an inverted Olympus BX50WI microscope with a KAI-1001 S/N 5B7890-4201 Olympus camera attached. The fluorescence ratiometric images from data obtained at excitation wavelengths of 340 and 380 nm were viewed and analysed using OraCal pro, Merlin morphometry temporal mode (Life Sciences resources, Version 1.20). The DRG neurones using OraCal pro, Merlin morphometry temporal mode (Life Sciences resources, Version 1.20).

2.4. Evaluation of calcium channel subunit expression by quantitative TaqMan PCR

Populations of cultured DRG neurones and F-11 cells which either responded to gabapentin or were found to have low sensitivity or to be insensitive to gabapentin were treated with triazol and the resulting solution was rapidly frozen in sterile tubes on dry ice. Total RNA was isolated from DRG cultures using the RNeasy isolation method (Qiagen). Cell lysates were homogenised using a QIAshredder (Qiagen) prior to RNA purification. Total RNA was isolated from F11 cells using the Trizol method (Life Technologies). Total RNA was treated with DNase I (Life Technologies) to remove any contaminating genomic DNA. Prior to reverse transcription each sample was tested for genomic contamination using mRNA specific TaqMan quantitative PCR (reagents from Applied Biosystems). DNA-free RNA samples (1–2 µg) were pelleted and reverse transcribed in a 20 µl reaction using Superscript II reverse transcriptase (Life Technologies) with an anchored oligo dT primer. The relative abundance of voltage dependent calcium channel mRNAs were assessed by TaqMan quantitative PCR, in addition four separate ‘housekeeping genes’ were used as references to normalise the data. Five nanogram total RNA equivalents were used for each 25 µl TaqMan reaction using 900 µM each primer and 100 nM probe in 1×TaqMan Universal Master Mix (Applied Biosystems). Reactions were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The following primers and probes were used to measure the abundance of each RNA species. Each TaqMan probe was modified with a 5’ reporter dye (FAM, 6-carboxy fluorescein) and a 3’ quencher dye (TAMRA, 6-carboxytetrathymethylrhodamine) which where synthesised by Applied Biosystems: α-1-F GAGATGACACAGCGGACTTC, α-1-R CACCCCTTTGCGATTTTGATA, α-1-T TaqMan probe: TGGCTCTGATCGGAAGGCCC (from Genbank M64373); α-β-F GACAAGGGCAACGGTGCTCTAGA, α-β-R CAAGTACGGTACGAGTGTATGTTT, α-β-T TaqMan probe: CCACGCTCTCGCCGGAAA (from Genbank AF055477); α-γ-F AGCACTTCCCTCTAGACGTTTG, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG, α-γ-F AGCAGCAAGACGAGTC, α-γ-R CTGTTACCACCAGCGAGATGC, α-γ-T TaqMan probe: CCACCCTTCAAGAGATTG.
2.4.1. Data analysis:

\[\Delta C_T = \frac{C_T \cdot \text{DNA}}{C_T \cdot \text{DNA} + \text{Probes}}\]

\[\Delta CT = \text{CT} \cdot \text{Target} - \text{CT} \cdot \text{Reference}\]

\[\text{Fold} = 2^{\Delta C_T}\]

2. Results

3.1. Actions of gabapentin on the electrophysiological properties of cultured neonatal rat DRG neurones, comparisons with GABA

Initial experiments under current clamp conditions were carried out to compare the actions of GABA and gabapentin (Fig. 1(A)). Gabapentin (2.5 µM) produced no significant change in membrane potential or input resistance. In contrast, GABA (10 µM) reversibly depolarised the resting membrane potential by 25 mV and reduced the input resistance by 213 mΩ (n=10, P<0.0001; Student’s t-test; Fig. 1(B)). This was consistent with the activation of a GABA-activated Cl− conductance and a Cl− equilibrium potential close to 0 mV. Gabapentin did not produce any consistent or significant changes in the properties of evoked single action potentials activated from a potential of −70 mV by 5 ms depolarising current step commands (n=13). However, it was noted that gabapentin reduced action potential after-depolarisations (n=4) an effect that was indicative of reduced activation of Ca2+-activated chloride conductances. To evaluate the actions of gabapentin on Ca2+ channel conductances prolonged action potentials were variable even in single neurones held at −70 mV and stimulated consistently at a low frequency of once every 60 s. In spite of considerable variability gabapentin (2.5 µM) significantly reduced the duration of Ba2+ action potentials by 71±7% (n=10, P<0.01, Fig. 2). A more detailed study of this inhibition of Ca2+ channel conductances was undertaken under voltage clamp conditions to obtain more reliable quantitative data.

Application of gabapentin to cultured DRG neurones obtained using method 1 and cultured in the presence of 20 ng/ml NGF and 10% serum resulted in modest but significant dose-dependent inhibition of voltage-activated Ca2+ currents. Gabapentin (0.25–25 µM) reversibly attenuated high voltage-activated (HVA) Ca2+ currents evoked from a holding potential of −90 mV by 100 ms voltage step commands to 0 mV (Fig. 3(A)). At a concentration of 2.5 µM pressure ejection of gabapentin for 3 min gave the maximum inhibition of 23±2 and 35±4% (mean±s.e.m. n=16) for the mean Ca2+ current measured at the peak and at the end of the voltage step command, respectively. The inhibition produced by gabapentin of the mean peak Ca2+ current and the current measured at the end of the 100 ms voltage step commands were not significantly different (Fig. 3(B)). The response to gabapentin reversed over a 3–5 min recovery period. The voltage-dependence of the inhibitory action of gabapentin was investigated in three ways, a double pulse protocol, comparing inhibitory action at holding...
potentials of $-90$ and $-40\, \text{mV}$ and by comparing $V_{0.5}$ values for apparent steady state inactivation. The double pulse protocol involved holding the neurones at $-90\, \text{mV}$ applying pre-pulses to $+100\, \text{mV}$ for 30 ms and then returning to $-90\, \text{mV}$ for 15 ms prior to activating test voltage step commands to 0 mV (Fig. 4(A)). This protocol was applied under control conditions in the absence of drug and after gabapentin had produced steady state inhibition of the $\text{Ca}^{2+}$ current. Mean $\text{Ca}^{2+}$ current amplitudes from six experiments with and without the pre-pulses were compared under the two experimental conditions. The double pulse protocol had no significant effect on $\text{Ca}^{2+}$ currents recorded under control conditions. However, in the presence of gabapentin the inhibition of the peak $\text{Ca}^{2+}$ current showed a modest but significant additional voltage-dependent block when the pre-pulse protocol was applied (Fig. 4(A)). A voltage-dependent component of gabapentin action was also revealed when gabapentin evoked greater inhibition of the $\text{Ca}^{2+}$ currents activated from a holding potential of $-40\, \text{mV}$ compared with currents activated from a holding potential of $-90\, \text{mV}$. $\text{Ca}^{2+}$ currents activated at 0 mV were inhibited by $44\pm8\%$ compared to $25\pm4\%$ ($n=7$, $P<0.05$; Student’s $t$-test, Fig. 4(B)), for data obtained from holding potentials of $-40$ and $-90\, \text{mV}$, respectively. Furthermore, plotting complete steady state inactivation curves for currents activated from holding potentials between $-100$ and $-10\, \text{mV}$ showed that gabapentin produced a shift to the left in this relationship. The apparent $V_{0.5}$ values for inactivation (voltage at

Fig. 1. (A) The structures of GABA and gabapentin. (B) Bar chart showing the mean values for resting membrane potential and input resistance, (measured from hyperpolarising electrotonic potentials) under control conditions, during application of gabapentin (2.5 $\mu\text{M}$) and during the peak of the response to GABA (10 $\mu\text{M}$). Inset record shows resting potential and electrotonic potentials during an experiment carried out to compare the effects of gabapentin and GABA.
Fig. 2. Gabapentin reduced the duration on Ba$^{2+}$ action potentials. Bar chart showing mean (±s.e.m.) action potential durations normalised with respect to the first stable control, under control conditions (Control 2; NS from Control 1) and during the application of gabapentin (2.5 µM) (gabapentin 1 and 2, 60 s apart). Inset records show variable prolonged action potentials recorded from two DRG neurones and the inhibitory action of gabapentin. The neurones were held at −70 mV and Ba$^{2+}$ action potentials were evoked by 5 ms depolarising current commands.

which half the functional channel population are available to open at a test voltage of 0 mV) were −49.9±4.9 mV under control conditions and −57.2±4.2 mV in the presence of 2.5 µM gabapentin (n=9; P<0.02).

In this study the actions of gabapentin were not dependent on the frequency of Ca$^{2+}$ current activation. Increasing the frequency of Ca$^{2+}$ current activation from 0.033 to 0.1 Hz (n=5) did not increase the gabapentin-evoked steady state inhibition (Fig. 4(C)).

Previously, we have investigated the pharmacology of the gabapentin-sensitive Ca$^{2+}$ current (Sutton et al., 2002). Nifedipine, ω-CgTx GVIA and ω-Aga IVA were used and our data suggested that there is considerable overlapping pharmacology with reduced sensitivity to gabapentin being observed with all of the above compounds. We, therefore, decided to take another approach to investigate the involvement of L-type Ca$^{2+}$ channels and use Bay K8644 to enhance L-type current. The protocol used was to evoke inhibition of Ca$^{2+}$ currents with 2.5 µM gabapentin and then in the continual presence of gabapentin apply Bay K8644 (1 µM in 0.01% ethanol). In six experiments significant inhibition of the Ca$^{2+}$ current was observed with gabapentin alone but subsequent application of Bay K8644 with gabapentin in all cases resulted in enhancement of the Ca$^{2+}$ current back towards control levels (Fig. 5).

Fig. 3. Gabapentin produced modest but significant reversible inhibition of high voltage-activated Ca$^{2+}$ currents. (A) Graph showing the dose-response relationship for gabapentin and percentage inhibition for Ca$^{2+}$ currents measured at the peak inward current level and at the end of a 100 ms voltage step command to 0 mV. Inset voltage and Ca$^{2+}$ current records show the partial reversal of the gabapentin response after 5 min. (B) Bar graph showing the inhibitory actions of gabapentin on the mean peak Ca$^{2+}$ current and the current measured at the end of the 100 ms voltage step commands. The control values are significantly different from current values (filled bars) obtained during application of gabapentin. The hatched bars show the mean±s.e.m. values for the current inhibited by gabapentin. Inset voltage and inward current records showing the inhibitory actions of gabapentin on a Ca$^{2+}$ current and the difference current inhibited by gabapentin.
Gabapentin inhibition of Ca²⁺ channel currents was increased by depolarisation. (A) Bar chart showing data from double pulse experiments reveal the voltage-dependent increase in gabapentin-evoked inhibition of the peak (filled bars) high voltage-activated Ca²⁺ currents. A depolarising pre-pulse to +100 mV did not significantly alter control Ca²⁺ current values. The apparent additional gabapentin-evoked inhibition of the current measured at the end of a 100 ms voltage step command was not significant. Inset records show the voltage protocols used and the modulation of the Ca²⁺ currents by pre-pulse and gabapentin. (B) Bar chart showing the significant increase in the percentage inhibition produced by depolarising the holding potential from −90 mV to −40 mV with currents activated at 0 mV. (C) Line graph showing stable control peak Ca²⁺ currents activated every 10 s and the inhibition of peak Ca²⁺ current by gabapentin. Mean data ± s.e.m. (n=5). Inset records show voltage and Ca²⁺ current traces. The first and last control, inhibition by gabapentin and 3 min recovery.

3.2. Inhibition of calcium influx into cultured F-11 cells by gabapentin

We have previously shown that in Ca²⁺ imaging experiments on cultured DRG neurones, pre-treatment of the cultured cells with 25 µM gabapentin for 1–2 min prior or just during the depolarising stimuli of high K⁺ attenuated the Ca²⁺ influx. In DRG neurones gabapentin (25 µM) reversibly reduced the duration of the Ca²⁺ transients and the total Ca²⁺ influx evoked by application of NaCl-based recording medium containing 30 mM KCl. However, gabapentin did not significantly influence the mean peak amplitude of the Ca²⁺ transients in cultured DRG neurones and experiments using DRG neurones were limited under control conditions to only three consistent intracellular Ca²⁺ responses (Sutton et al., 2002). More consistent and repeatable data were obtained with the F11 cells (passaged five times or less) than with the cultured DRG neurones (Fig. 6(A)). Pre-treatment of F11 cells with gabapentin for 1–2 min before application of depolarising medium resulted in 11 ± 3 and 18 ± 2% (n=32 from four cultures P<0.005; P<0.0001) reductions in the Ca²⁺ transient amplitude and duration at 50% of transient amplitude respectively (Fig. 6(B) and (C)). Measuring the area under the curve of the Ca²⁺ transient to obtain the total Ca²⁺ flux also revealed the
3.3. The influence of culture conditions on the sensitivity of cultured cells to gabapentin

Time in culture, using method 1 to obtain the DRG neurones (with serum and 20 ng/ml 2.5S-NGF), did not significantly alter the inhibition of voltage-activated Ca\(^{2+}\) currents produced by gabapentin (2.5 µM). DRG neurones of increasing age in culture, from 3 h to 13 days in culture were used. The percentage inhibition of Ca\(^{2+}\) current produced by 2.5 µM gabapentin did not change over these periods in culture (Fig. 7).

However, culture conditions used to maintain DRG neurones and particularly the serum content and concentration of NGF applied to the neurones appeared to influence sensitivity to gabapentin. Whole-cell patch clamp recordings of the peak HVA Ca\(^{2+}\) channel current carried by Ba\(^{2+}\) (2–5 mM BaCl\(_2\), \(I_{\text{Ba}}\)) were undertaken on DRG neurones cultured in media 1 (serum and 10 ng/ml NGF-2.5s) vs 2 (100 ng/ml NGF-7s and serum-free) after 48 h. As shown by a previous study from our group (Sutton et al., 2002), gabapentin produced a relatively high affinity block of the peak HVA \(I_{\text{Ba}}\) recorded from media 1 DRG neurones. Bath application of gabapentin (25 µM) for 10 min produced a significant decrease in the peak HVA \(I_{\text{Ba}}\) (44.7 ± 4.1%, \(n=8\)). In contrast, however, DRG neurones maintained in a defined media (media 2, for more than 48 h) were significantly less sensitive to inhibition by gabapentin (18.5 ± 2.7, \(n=9, P<0.001;\) Fig. 8). Differences in cell phenotype could be determined visually, as cells maintained in media 2 developed larger cell bodies which could be quantified electrophysiologically in terms of their measured capacitance (20.1 ± 1.6 vs 29.1 ± 1.9 pF, \(n=39, 22\) for media 1 vs 2, respectively; \(P<0.0001;\) Fig. 8).

These results with gabapentin on different populations of DRG neurones and differentiated F-11 cells prompted significant and clear inhibitory action of gabapentin on the voltage-dependent Ca\(^{2+}\) flux (Fig. 6(D)).

Changes in sensitivity to gabapentin were also observed in Ca\(^{2+}\) imaging studies on differentiated F-11 cells. Responses to gabapentin were only observed in differentiated F-11 cells with low passage numbers and not cells passaged more than five times (Fig. 6(E)).
us to compare the $\text{Ca}^{2+}$ channel subunits expressed by each population of gabapentin-sensitive and insensitive DRG neurones, even though we might gain more of an insight into where inhibitory interaction of gabapentin take place on the subunit complexes of voltage-activated $\text{Ca}^{2+}$ channels.

3.4. Subunit expression data for DRG neurones and F-11 cells.

TaqMan analysis was undertaken to gain an insight into any differences in $\text{Ca}^{2+}$ channel subunit expression of the gabapentin-sensitive and insensitive DRG neurones. As shown in Table 1, the expression of $\beta_3$ was 4.58 fold higher in the media 1 culture conditions compared with the media 2 cultures. In contrast, a 2.21 fold elevation in $\alpha_\delta$-2 expression was observed in the media 2 cultures compared with the media 1 cultures.

TaqMan analysis of $\text{Ca}^{2+}$ channel subunit expression in the F-11 cells, which responded to gabapentin, compared with non-responsive F-11 cells revealed a similar trend to that seen in DRG neurones. A 16.68 (1.86–149.37) fold higher expression of $\beta_2$ was observed in the F11 cells that responded to gabapentin compared with 1.00 (0.65–1.54) in the non-responders. In addition, we observed a 4.31 (2.84–6.52) fold increase in $\alpha_\delta$-2 expression in the gabapentin insensitive F-11 cell cultures relative to 1.00 (0.69–1.45) for F-11 cells that responded to gabapentin. No detectable changes were observed in any of the other voltage-activated $\text{Ca}^{2+}$ channel subunits. Therefore, for both cultured DRG neurones and F-11 cells sensitivity of $\text{Ca}^{2+}$ channels to inhibition by gabapentin was associated with relatively low $\alpha_\delta$-2 subunit expression and higher $\beta_2$ subunit expression compared with cells which had low sensitivity to gabapentin or appeared not to respond.

3.5. Signalling data with PTX-treated neurones and a PKA activator and PKA inhibitor

Previously, the GABAB receptor agonist baclofen was found to inhibit $\text{Ca}^{2+}$ channel currents carried by $\text{Ba}^{2+}$ in DRG neurones in a PTX-sensitive manner (Dolphin and Scott, 1987). Cultured DRG neurones pre-treatment with PTX may have provided an approach to investigate and exclude the possibility that GABAB receptors are a target site for gabapentin-evoked inhibition of HVA $\text{Ca}^{2+}$ channels. Surprisingly, 2.5 $\mu\text{M}$ gabapentin failed to significantly inhibit voltage-activated $\text{Ca}^{2+}$ currents (n=8) in

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### Table 1

Voltage-activated $\text{Ca}^{2+}$ channel subunit expression data for DRG neurones (*significantly different (P<0.05)). Pooled data from four separate media 1 and 2 culture comparisons. Relative expression was calculated using the equation $2^{-\Delta C_T}$, where $\Delta C_T$ represents the subtraction by the $C_T$ calibrator value. The values in brackets represent the range, calculated by $2^{-\Delta C_{T} \pm s}$, where $s$ is the standard deviation of the $\Delta C_T$ value. The expression of each VDCC subunit was analysed independently of each other and normalised against the housekeeping gene cyclophilin. The relative expression values were set to 1.00 for the lowest level of expression in each pair-wise comparison. The data from these four comparisons were pooled and tested for statistical significance. Expression of $\alpha_E$, $\alpha_F$, $\alpha_G$, $\alpha_I$, $\alpha_S$ and $\gamma-1$ was undetectable in these cultures.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Relative expression of VACC subunits in media 1 DRG cultures</th>
<th>Relative expression of VACC subunits in media 2 DRG cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_A$</td>
<td>1.04 (0.87–1.25)</td>
<td>1.60 (1.35–1.90)</td>
</tr>
<tr>
<td>$\alpha_B$</td>
<td>1.06 (0.84–1.32)</td>
<td>1.77 (1.28–2.44)</td>
</tr>
<tr>
<td>$\alpha_C$</td>
<td>1.10 (0.94–1.29)</td>
<td>1.77 (1.54–2.04)</td>
</tr>
<tr>
<td>$\alpha_D$</td>
<td>1.00 (0.76–1.32)</td>
<td>1.53 (1.19–1.97)</td>
</tr>
<tr>
<td>$\alpha_H$</td>
<td>1.16 (0.95–1.42)</td>
<td>1.54 (1.36–1.74)</td>
</tr>
<tr>
<td>$\alpha_{\delta-1}$</td>
<td>1.12 (0.95–1.31)</td>
<td>1.32 (1.14–1.53)</td>
</tr>
<tr>
<td>$\alpha_{\delta-2}$</td>
<td>1.00 (0.78–1.29)</td>
<td>2.21 * (1.52–3.20)</td>
</tr>
<tr>
<td>$\alpha_{\delta-3}$</td>
<td>1.32 (1.08–1.61)</td>
<td>1.76 (1.39–2.23)</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>1.28 (1.05–1.55)</td>
<td>1.27 (1.06–1.52)</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>4.58* (2.49–8.43)</td>
<td>1.03 (0.79–1.35)</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>1.11 (0.99–1.25)</td>
<td>1.33 (1.00–1.77)</td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>1.30 (1.08–1.57)</td>
<td>1.72 (1.35–2.20)</td>
</tr>
</tbody>
</table>
DRG neurones pre-treated with PTX (Fig. 9). However, a role for GABA<sub>B</sub> receptors in mediating the actions of gabapentin on high voltage-activated Ca<sup>2+</sup> channels was excluded by experiments carried out in the continued presence of GABA<sub>B</sub> receptor antagonists. Cultured DRG neurones continually exposed to the GABA<sub>B</sub> receptor antagonist, saclofen (20 µM), remained sensitive to inhibition by gabapentin (22±3%, n=10, P<0.05; Fig. 9).

The role of PKA-dependent activity was assessed by pre-incubating with the specific PKA inhibitor, Rp-CAMPS (30 µM), or the specific PKA activator, Sp-CAMPS (30 µM). The results shown in Fig. 10 indicate that PKA activity mediates the effect of gabapentin to a differing extent in both sets of cells. Inhibition by gabapentin in both media 1 and 2 cells was significantly reduced following a 30 min pre-incubation with Rp-CAMPS — indicating a basal, PKA-dependent up-regulation of the gabapentin-sensitive current (4.8±4.6 and 12.6±6.9%, n=6, 6 for media 1 and 2, respectively). Moreover, in both sets of neurones the effects of gabapentin were enhanced by maximally activating PKA above basal levels using Sp-CAMPS (69.6±4.6 and 52.5±8.9%, n=6, 8 for media 1 and 2, respectively). Nevertheless, the gabapentin-sensitive current in media 2 cells where PKA was maximally activated was still less than that observed for media 1 cells, suggesting that both basal levels of PKA activity and potential PKA-sensitive targets are decreased in media 2 cells.

4. Discussion

High voltage-activated Ca<sup>2+</sup> currents were attenuated by gabapentin in cultured DRG neurones as previously observed for Ba<sup>2+</sup> currents (Sutton et al., 2002). A modest difference between our two studies being in the efficacy of gabapentin action, which most likely related to the different methods and durations of drug application used. In this study the responses were reversible and consistent results were obtained using whole cell patch
clamp recording, Ca$^{2+}$ imaging and F-11 cells. A degree of voltage-dependent inhibition of Ca$^{2+}$ currents by gabapentin was seen in this study. The low level of additional block seen with a depolarising pre-pulse was distinct from the action of gabapentin on Ca$^{2+}$ currents in cortical pyramidal neurones (Stefani et al., 1998). However, in our study no voltage-dependent facilitation of control currents was observed in the DRG neurones and only the peak current was significantly affected in the presence of gabapentin by the double-pulse protocol, suggesting differences in tonic modulation of the Ca$^{2+}$ channels in the two preparations. Previously, it has been shown that a double-pulse protocol was required to evaluate gabapentin-mediated inhibition of Ca$^{2+}$ currents in a DRG neurone preparation and that attenuation of the currents also occurred in a use-dependent manner (Alden and Garcia, 2001). Although we observed a more modest voltage-dependence we used a shorter pre-pulse and a lower concentration of gabapentin which may have limited the voltage-dependent effect, which was clearer in the study by Alden and Garcia (2001). However, in the present study we did not observe a use-dependent component to inhibition, which may be secondary to other mechanisms of block and at higher concentrations involve gabapentin interactions with the open channel.

Although in DRG neurones a major component of the current sensitive to gabapentin is sensitive to ω-CgTx GVIA, implicating N-type Ca$^{2+}$ channels as a primary target in some neurones the pharmacology is not clear cut. Data suggest that a variety of different α$_1$ subunits may be affected by gabapentin (Stefani et al., 1998; Fink et al., 2000). This adds a new level of complexity to pharmacological classification of Ca$^{2+}$ channels, which in the past has mainly been based on α$_1$ subunit–ligand interactions. Our previous data using pharmacological tools to identify different Ca$^{2+}$ current subtypes involved in gabapentin responses suggested that there is considerable overlapping pharmacology with reduced sensitivity to gabapentin being observed after application of nifedipine, ω-CgTx GVIA or ω-Aga IVA (Sutton et al., 2002). The data from this study suggested that gabapentin does not specifically interact with a particular channel subtype that can be characterised with classical pharmacological tools. This suggestion contrasts with the finding that L-type channels are the predominant targets for gabapentin in cortical pyramidal neurones (Stefani et al., 1998). In the present study we have carried out some additional experiments with the 1, 4-dihydropyridine Bay K 8644, which enhances current flow through L-type Ca$^{2+}$ channels. Bay K8644 enhanced the Ca$^{2+}$ current, which remained in the continual presence of gabapentin. The two most likely possible reasons for this are: first, that Bay K8644 may displace gabapentin from its binding site, but to date there is no evidence for such an interaction; secondly, that Bay K8644 enhances current flow through L-type channels that are resistant to gabapentin. This latter explanation, is the one we most favour because although nifedipine pre-treatment did attenuate gabapentin responses they were not abolished in DRG neurones (Sutton et al., 2002). Together the data from these studies suggest that channels in addition to L-type Ca$^{2+}$ channels were involved in gabapentin responses and that some L-type channels were resistant to gabapentin and available for modulation by Bay K8644. Clearly, apparently conflicting data has been obtained from cultured DRG and cortical pyramidal neurones but this may be explained by different preparations expressing distinct α$_1$ subunits in combination with α$_2$δ and other subunits.

A key finding of this study was that some preparations of DRG neurones and F-11 cells did not respond to gabapentin as well as others and this related to culture conditions and in the case of F-11 cells to passage number. Serum and neurotrophin content of the culture medium appear to be important influences on gabapentin responsiveness of DRG neurones. A number of studies have shown that Ca$^{2+}$ channel expression is altered but not always similarly by neurotrophins such as NGF (Usowicz et al., 1990) and recently work on developing hippocampal neurones has shown neurotrophins selectively up-regulate specific Ca$^{2+}$ channel types by a Trk receptor/MAP-kinase pathway (Baldelli et al., 2000). Analysis of the relative Ca$^{2+}$ channel subunit expression identified in both cell types, DRG neurones and F-11 cells, showed two consistent trends. First, sensitivity to gabapentin was associated with relatively higher levels of β$_2$ Ca$^{2+}$ channel subunit expression. Secondly, sensitivity to gabapentin was associated with relatively lower α$_2$δ-2 Ca$^{2+}$ channel subunit expression. This may explain why some studies show sensitivity of HVA neuronal Ca$^{2+}$ currents to gabapentin and some do not, but more importantly it sheds further light on the molecular interactions between subunits which lead to gabapentin inhibition of Ca$^{2+}$ channels. Our data are open to several interpretations. Part of the α$_2$δ subunit, which provides a high affinity-binding site for gabapentin (Gee et al., 1996), is extracellular. It has also been shown that deletion mutants, which lack the delta polypeptide and varying sections of the α$_2$ element, failed to bind gabapentin and residues in the δ polypeptide, critical for gabapentin binding have been identified (Brown and Gee, 1998). Functionally along with β subunits, α$_2$δ subunits increase peak Ca$^{2+}$ current amplitude (Shistik et al., 1995; Dolphin et al., 1999) and now these accessory subunits also appear in influence pharmacological properties of the channels. It may be that gabapentin differentially interacts with the different α$_2$δ subunits in DRG neurones. This has been shown although it appears that the type 2 α$_2$δ subunit has a high affinity for gabapentin compared with the doses of drug used in this study (Marias et al., 2001). Nevertheless changes in the relative proportions of type 1 and type 2 α$_2$δ subunit could
account for a change in sensitivity of preparations to gabapentin. Additionally, the functional influence of gabapentin on the Ca\(^{2+}\) channel current is not confined to interactions with \(\alpha\delta\) subunits but also appears to involve the \(\beta\) subunit expressed. Co-operative interactions between accessory subunits may, therefore, be critical to gabapentin influencing currents passing through different \(\alpha\) subunits of different Ca\(^{2+}\) channels.

Recently three distinct types of \(\alpha\delta\) subunit have been identified, type 3 did not bind gabapentin, however, types 1 and 2 bound gabapentin with high affinity with \(K_d\) values of 59 and 153 nM, respectively (Marias et al., 2001). Brain tissue expressed all three types of \(\alpha\delta\) subunit but at present the expression of these calcium channel subunits in DRG neurones is unknown. Multiple types of \(\alpha\delta\) subunit add an additional level of complexity to the analysis of gabapentin actions. A further important recent discovery is that \(\alpha\delta\) subunit expression changes in neuroplasticity and this is a specific feature in DRG neurones during the development of allodynia (Lou et al., 2001). Thus changes in gabapentin sensitivity may result from events associated with distinct types of injury and disease states and changes in \(\alpha\delta\) subunit expression is one of several mechanisms likely to be involved. It has now been shown using a tactile allodynia model that with upregulation of \(\alpha\delta\) subunit expression, gabapentin can reverse the allodynia state in a dose-dependent manner as assessed by measuring paw withdrawal threshold to mechanical stimulation (Lou et al., 2001).

GABA receptor chloride channel complexes expressed on DRG neurones appear not to be critical for gabapentin responses. As previously reported gabapentin did not mimic the effects of GABA on membrane potential. We originally had expected that gabapentin might act on PTX-pre-treated DRG neurones and thus rule out the involvement of GABA\(_B\) receptors. However, gabapentin responses were attenuated by PTX-pre-treatment, which may implicate a G-protein coupled receptor and a GABA\(_B\) receptor in particular. This is particularly relevant given the findings of Ng et al. (2001) who have reported that GABA\(_B\) receptors with specific heterodimeric composition are the target site through which gabapentin works to activate inwardly rectifying K\(^+\) conductances and thus reduce neuronal excitability. In this present study experiments were carried out in the presence of the GABA\(_B\) receptor antagonist saclofen and gabapentin continued to inhibit Ca\(^{2+}\) currents suggesting that at least modulation of Ca\(^{2+}\) channels in DRG neurones is independent of GABA receptor activation. The issue of why gabapentin responses are sensitive to PTX remains to be addressed. The importance of the \(\beta\) subunits of Ca\(^{2+}\) channels for G-protein modulation of Ca\(^{2+}\) channels is well established (Dolphin, 1998). The results obtained in this study may relate to the importance of Ca\(^{2+}\) channel accessory subunits and precoupling of G-protein linked receptor-channel complexes which may be utilised by gabapentin but disrupted by PTX.

A further finding was that the modulation of PKA with agonists and antagonists also influenced the action of gabapentin on Ca\(^{2+}\) channel currents. This raises an important topic of channel phosphorylation and drug sensitivity. From our study we would predict that a level of PKA-mediated phosphorylation is important for gabapentin responsiveness although it remains to be determined how this fits in with Ca\(^{2+}\) channel subunit expression. Subunit phosphorylation may influence channel availability and Ca\(^{2+}\) channel subunit interactions which in turn determine the action of gabapentin.

We conclude that the expression of accessory Ca\(^{2+}\) channel subunits and specifically \(\beta_1\) and \(\alpha\delta\)-2 subunits influences the modulation of high voltage-activated Ca\(^{2+}\) channels by gabapentin. There is clear potential for gabapentin to influence pain transmission by an inhibitory action on Ca\(^{2+}\) channels expressed on sensory neurones as well as neurones in the CNS. Interestingly, it has recently been shown that chronic gabapentin treatment of immature rats (kainic acid seizure model) had no long-term harmful effects on cognitive functions during development (Cilio et al., 2001). This suggests that the mechanisms available for the action of gabapentin can be specific to particular situations. The subunit composition of Ca\(^{2+}\) channels on neurones involved in a variety of functions including transmitting and processing sensory information may change depending on local factors and influences associated with disease states and injury. Therefore, Ca\(^{2+}\) channel subunit expression may provide a mechanism by which the effectiveness of gabapentin is altered and particular culture conditions may result in sensory neurones taking on particular phenotypes associated with injury or disease states.

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