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Recombinant $GABA_B$ receptors formed from $GABA_{B1}$ and $GABA_{B2}$ subunits selectively inhibit N-type Ca^{2+} channels in NG108-15 cells

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Abstract

Efficient transfection of NG108-15 cells with $GABA_B$ receptor subunits was achieved using polyethylenimine. Baclofen modulated high voltage-activated Ca^{2+} current in differentiated cells transfected with $GABA_{B1}$ and $GABA_{B2}$ receptor subunits or with the $GABA_{B2}$ subunit alone, but not with the $GABA_{B1}$ subunit alone. Characteristics of the current modulation were very similar for cells transfected with $GABA_{B1/2}$ and $GABA_{B2}$ subunits. Using antisense oligonucleotides against $GABA_{B1}$ subunits and also western immunoblotting, we are able to show that NG108-15 cells contain endogenous $GABA_{B1}$ subunits. Therefore, functional receptors can be formed by the combination of native $GABA_{B1}$ subunits with transfected $GABA_{B2}$ subunits, in agreement with the proposed heteromeric structure of $GABA_B$ receptors. Finally, we used selective channel blockers to identify the subtypes of Ca^{2+} channels that are modulated by $GABA_B$ receptors. In fact, in differentiated NG108-15 cells, the recombinant $GABA_B$ receptors couple only to N-type Ca^{2+} channels.

Keywords: $GABA_B$ receptor; Ca^{2+} current; Ca^{2+} channel subtype; Transfection; NG108-15 cell; Antisense

1. Introduction

The metabotropic receptor for γ-aminobutyric acid (GABA), $GABA_B$, exerts an important influence upon synaptic signalling in the central nervous system by modulating ion channel function (reviewed by Kerr and Ong, 1995). Activation of presynaptic $GABA_B$ receptors leads to a reduction in the release of neurotransmitter predominantly via inhibition of voltage-gated Ca^{2+} channels (Scholz and Miller, 1991; Doze et al., 1995; Huston et al., 1995; Takahashi et al., 1998). Postsynaptic receptors couple in a positive manner to K^+ channels, which underlies the slow postsynaptic hyperpolarisation induced by GABA (Soltesz et al., 1988; Dutar and Nicoll, 1988; Lüscher et al., 1997).

As a result of the cloning of $GABA_B$ receptor genes, some more of the details of the molecular mechanisms, which contribute to these behaviours, have been characterized. The hypothesis that the functional receptor is a heterodimer of two subunits, $GABA_{B1}$ and $GABA_{B2}$, is strongly supported by available evidence (reviews by Bowery and Enna, 2000; Couve et al., 2000). Ion channel modulation by the recombinant receptors ($GABA_{B1}$/$GABA_{B2}$ subunit combinations) have largely focused on K^+ channels in non-neuronal systems (Jones et al., 1998; White et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999). Recently, however, the inhibition of voltage-gated Ca^{2+} channels by activation of recombinant receptors formed by co-transfection of $GABA_{B1}$ and $GABA_{B2}$ subunits has been described in postganglionic sympathetic neurones (Filippov et al., 2000) as well as in the NG108-15 cell line, which is derived partly from a neuroblastoma cell line. In the primary neurones, though, activity of endogenous $GABA_B$ receptors is apparent. In apparent contradiction to the suggestion of heterodimer formation, there is evidence that the $GABA_{B2}$ subunit may be active in the absence of $GABA_{B1}$ subunits (Martin et al., 1999). Therefore, one aim of the present study is to identify the $GABA_B$ subunit combination that is able to modulate voltage-gated Ca^{2+} channels using the NG108-15 cell line, which does not contain functional $GABA_B$ receptors. We have previously published a preliminary account of the function of recombinant $GABA_B$ receptors in NG108-15 cells (Easter and Spruce, 2000), though problems with the transfection methodology severely limited interpretation of the findings.

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We also investigate the coupling of the recombinant receptors to different voltage-gated Ca^{2+} channel subtypes, which are endogenously expressed in differentiated NG108-15 cells. Those which couple to native $GABA_B$ receptors are primarily of the N- and P/Q-types, but there is also evidence that L-type channels can be modulated (Scholz and Miller, 1991; Mintz and Bean, 1993; Menon-Johansson et al., 1993; Amico et al., 1995).

2. Materials and methods

2.1. Cell preparation

NG108-15 cells were grown in Dulbecco's modified Eagles medium (DMEM) with either 5% or 10% foetal bovine serum, 0.1 mM hypoxanthine, 0.4 μM aminopterin and 160 μM thymidine (HAT). For recordings, cells were transferred on day 0 to 35-mm petri dishes in DMEM+1% foetal bovine serum (plating medium), transfected (see below) and differentiated using plating medium with 10 μM prostaglandin E_1 and 50 μM isobutyl-1-methylxanthine (IBMX). Penicillin (120 units/ml) and streptomycin (12 ng/ml) were also included. On day 3, the differentiation medium was replaced with DMEM plus 1% foetal bovine serum. Recordings were made on days 3–7.

2.2. Transfection

Plasmid vectors containing cDNA encoding $GABA_{B1a}$ subunit (in pCDNA3-1(−)), $GABA_{B2}$ subunit (in pCDNA3) (GlaxoWellcome Pharmaceuticals, Stevenage, Herts, UK) and green fluorescent protein (GFP; in pEGFP-N1, Clontech) were amplified using standard molecular biology techniques. NG108-15 cells were transfected either with green fluorescent protein plasmid alone (control) or with this plasmid plus at least one of the $GABA_B$ subunit plasmids using the cationic polymer polyethylenimine (Sigma, 50% w/v). The transfection solutions were designed such that the final molar ratio of polyethylenimine amine nitrogen to DNA phosphate ratio was 10:1. Previous studies have shown that this is the optimum ratio for DNA transfection (Boussif et al., 1995).

Stock solutions of polyethylenimine (9 μg polyethylenimine/μl in RNase-free water, pH 7.0 with HCl) and NaCl (150 mM, filter sterilised) were prepared. Typically, ten 35 mm dishes were transfected with a particular plasmid combination. The amounts given are for one dish. Thus, 13 μl NaCl was added to each of two sterile eppendorfs. The required plasmid cDNAs (typically a total of 5 μg DNA/dish) were added to one eppendorf and polyethylemimine solution (0.3 μl/μg DNA) to the other. The solutions were mixed carefully and left for 10 min. The polyethylemimine solution was carefully added to the DNA solution, briefly vortexed and then left for a further 10 min. The polyethylemimine–DNA mixture was added to plating medium (0.8 ml/dish) to make up the final transfection solution, which was vortexed and left for 10 min before replacing the medium in the dish. The cells were incubated at 37 °C for 2–4 h. Then, the transfection solution was removed and replaced with differentiation medium. Using this methodology, approximately 10% of cells were successfully transfected.

2.3. Antisense treatment

Oligodeoxynucleotides (AltaBioscience, University of Birmingham) were included in the transfection solutions for some experiments (serum was not present). Two different antisense oligonucleotides were used, both of which are complementary to 18 base pair regions in the extracellular domain of the $GABA_{B1}$ subunit, over a range where the $GABA_{B1a}$ and $GABA_{B1b}$ splice variants are homologous but close to the point where they diverge. Each antisense had its own missense sequence to which it was compared for analysis. The sequences are as follows: ASI—TACTGCACGCCGTTCTGA; ASII—CTGCGGCTGTTAACGTCC; MSI—TCGCTCGACAGCTCGATC; MSII—AGCGCTCGGCTCGTCTTA. Phosphorothiorate-linked oligonucleotides were used and were dissolved in RNase-free distilled water to give a stock solution of 1 mM, which was kept at −20 °C until required.

2.4. Electrical recordings

Whole-cell Ca^{2+} current was recorded from fluorescent NG108-15 cells. Seals were made in a modified Kreb's solution containing (in mM): 120 NaCl, 10 HEPES, 11.1 glucose, 3 KCl, 1.5 $MgCl_2$, 2.5 $CaCl_2$ (adjusted to pH 7.4 with NaOH). Isolation of Ca^{2+} current was achieved by exchanging this solution for one containing 120 TEACl, 10 HEPES, 11.1 glucose, 3 KCl, 1.5 $MgCl_2$, 10 $CaCl_2$ (adjusted to pH 7.4 with NaOH). The pipette solution contained: 110 CsCl, 40 HEPES, 3 EGTA, 3 $MgCl_2$ (pH 7.4 with CsOH). During the experiment, the dish was continuously perfused with Krebs solution at a rate of 2–3 ml/min. The recording solution, to which various drugs were added, was applied directly to cells using a gravity flow system that included four separate reservoirs connected to a spritz electrode placed to within 100 μm of the cell (tip diameter 200 μm). The solution exchange time was estimated to be about 10 s, from observations of loss of sodium current. Current was measured using either a LIST L/M-PC or Axopatch 200A amplifier, filtered at 1 kHz and digitized at 6.25 kHz (pCLAMP, Axon Instruments). Cell capacitance and series resistance were read from the dials of the amplifier. Only recordings where series resistance was less than 10 MΩ were analysed (electrode resistance was 2–3 MΩ). In addition, series resistance compensation of 80% was routinely applied. The holding potential was set to −70 mV and Ca^{2+} current elicited by step depolarisations every 30 s. Leak and residual capacity currents were digitally removed using a P/-5 protocol.

The effect of drugs on Ca^{2+} current was assessed from voltage steps to +20 mV but the data was analysed in two different ways. For most experimental protocols, peak current was measured for each voltage step and expressed as a percentage of the average of three baseline values (obtained before drug application). However, for experiments using Ca^{2+} channel blocking agents, absolute changes in current density (obtained by dividing peak current by cell capacitance) were used to identify the channel subtypes modulated by baclofen. Large cells tend to have bigger current amplitude and since cell size is quite variable (assessed using capacitance), the current density calculation eliminates this variable. The total unblocked Ca^{2+} current could not be measured in all experiments, so this precluded using the percentage response to baclofen as a means of comparing treatment subgroups. For example, some cells were incubated in ω-conotoxin-GVIA for 15 min before washing it out and commencing recordings. In addition, the complexity of other experiments surpassed the capacity of our perfusion system (maximum of four different solutions) and therefore we had to begin recording in the presence of a channel-blocking agent. For all experiments, data were excluded if the baseline current variation was greater then 5%. For experiments using Ca^{2+} channel blocking agents, mean capacitance was 50.4 ± 4.6 pF ($n = 18$).

The proportion of total Ca^{2+} current carried via L-type channels was measured directly in cells where nifedipine was added during the recording. However, the contribution of N-type channels to the total Ca^{2+} current could only be estimated and the method that was used is described here. Experiments where current was preblocked using nifedipine, and ω-conotoxin-GVIA was added subsequently, were used for this analysis. The fractional amount of L-type current that had been blocked by nifedipine in these cells was assumed to be equal to that measured directly in separate cells (0.25: see Results). Therefore, if current in the presence of nifedipine is given as 1.0, that carried by all channel subtypes is calculated to be 1.33 (1.0/0.75). Hence, 0.33 is the current proportion carried via L-type channels. This is added to the fractional current that remains in ω-conotoxin-GVIA (r) to allow calculation of the proportion of N-type current, which is given by: $1-((r+0.33)/1.33)$.

All data is quoted as mean ± S.E.M. Statistical analysis was carried out using either analysis of variance (ANOVA) followed by a post hoc test or a t-test. Experiments were carried out at room temperature (18–22 °C).

2.5. Solutions

Drugs were made up as ×1000 stock solutions and were kept frozen (−20 °C) in aliquots until required. Noradrenaline (Sigma), ω-conotoxin-GVIA (Alomone, Jerusalem, Israel) and (−)baclofen (a gift from Dr W. Froestl, Novartis Pharma, Basel, Switzerland) were dissolved in dH_2O. Nifedipine (Sigma) was dissolved in dimethylsulfoxide (DMSO). Care was taken to ensure that noradrenaline- and nifedipine-containing solutions were protected from the light.

2.6. Western immunoblotting

Membranes from COS cells (African green monkey kidney cell line) as well as from NG108-15 cells (undifferentiated; differentiated; differentiated and transfected) were prepared, solubilised in 2× sample buffer and run on a 8.0% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane using a semidry transfer cell (Biometra) and blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline plus 0.05% Tween for 3 h. Membranes were incubated overnight at 4 °C with a primary antibody (5 μg/ml), which recognises the N-terminal domain of $GABA_{B1a}$ subunit (obtained from Dr M Pangalos, SmithKline Beecham; Filippov et al., 2000). Immunoreactivity was detected using a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase using the chemiluminescence SuperSignalkit (Pierce).

3. Results

The method of transfecting $GABA_B$ receptor subunits using polyethylenimine was successful. In initial experiments, NG108-15 cells were transfected with $GABA_{B1a}$ and $GABA_{B2}$ subunit plasmids (referred to subsequently as transfections of $GABA_{B1/2}$ subunits) as well as plasmid encoding green fluorescent protein and then differentiated. Recordings from fluorescent cells showed a robust and reversible inhibition of total Ca^{2+} current when exposed to 50 μM baclofen, a $GABA_B$ receptor agonist (Fig. 1). Cells transfected with plasmid encoding green fluorescent protein alone, however, showed no significant inhibition of Ca^{2+} current by baclofen ($P > 0.05$, ANOVA then Dunnet's test; Fig. 1). Nevertheless, the amount and voltage dependence of Ca^{2+} current for these transfection conditions were very similar as exhibited in current–voltage relations (Fig. 2).

Single subunit transfections were also carried out. Baclofen had no effect on Ca^{2+} current in cells transfected with the $GABA_{B1a}$ subunit (Fig. 1C). In contrast, $GABA_{B2}$ subunit-transfected cells did respond to baclofen, though the mean current inhibition was less than that seen in the double transfections (Fig. 1). In subsequent experiments, more detailed characterisation of the coupling of recombinant $GABA_B$ receptors to Ca^{2+} channels was carried out to enable comparison with native receptors. In particular, comparisons were made between the responses generated following transfections of $GABA_{B1/2}$ and $GABA_{B2}$ subunits to identify any possible discrepancies between receptors formed under these different conditions.

First, we wished to provide evidence that the modulation of Ca^{2+} current is indeed mediated by a $GABA_B$ receptor. The phosphinic acid derivative, 3-*N*[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*P*-benzyl-phosphinic

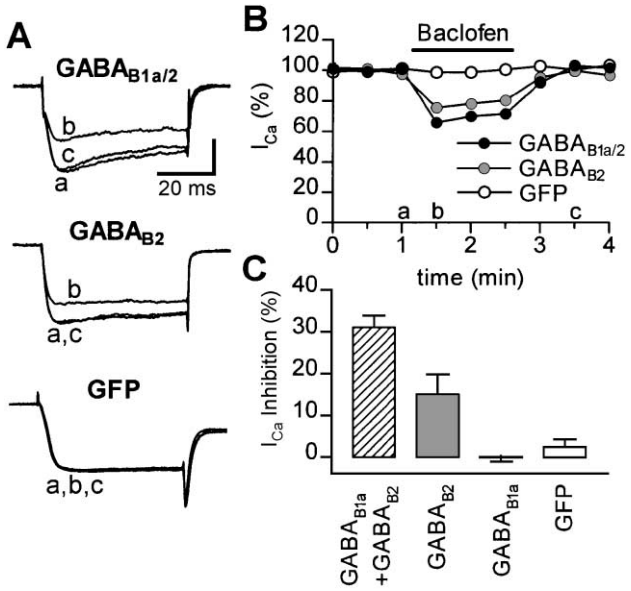

Fig. 1. Baclofen inhibits Ca^{2+} current in NG108-15 cells transfected with $GABA_{B2} \pm GABA_{B1a}$ subunits. (A) Current traces recorded in three different cells under the indicated transfection conditions and at +20 mV. GFP refers to cells transfected with plasmid encoding green fluorescent protein. For each cell, three traces are shown, which are extracts of the total recording and were obtained before, during and after 50 μM (−)baclofen exposure. The labels next to the traces refer to the time of recording (defined in part B). The vertical scale bar is 500 pA for the top two sets of traces and 1000 pA for the lower set. (B) Percentage Ca^{2+} current (I_{Ca}) is plotted against time for the three cells illustrated in A. Peak current during each trace is measured and the average current during the three baseline traces is defined as 100%. The bar indicates the duration of exposure to Baclofen. (C) Mean percentage inhibition of Ca^{2+} current by baclofen (I_{Ca} inhibition) for the indicated transfection conditions ($GABA_{B1a}$/$GABA_{B2}$, $n=30$; $GABA_{B2}$, $n=10$; $GABA_{B1a}$, $n=8$; GFP, $n=23$). The first trace during baclofen exposure is used to calculate the current inhibition.

acid (CGP 55845A), is a high-affinity and selective $GABA_B$ receptor antagonist, which has been shown to block baclofen-mediated inhibition of Ca^{2+} current in primary neurones (Bussieres and El Manira, 1999) as well as presynaptic

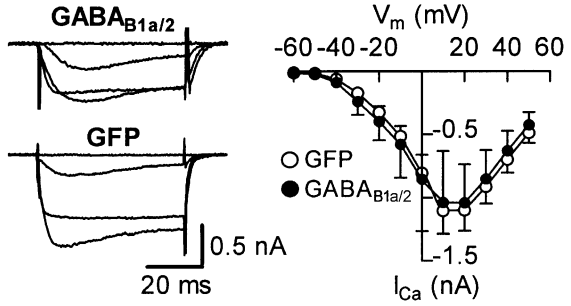

Fig. 2. Transfection of $GABA_B$ subunits does not alter characteristics of Ca^{2+} current. The two sets of traces show extracts of recordings from different cells, which had been transfected as indicated. The illustrated traces were recorded at −50, −20, 10 and 40 mV. The graph shows mean Ca^{2+} current (I_{Ca})−voltage relations for cells transfected with $GABA_{B1/2}$ subunits ($n=4$) and green fluorescent protein alone (GFP) ($n=9$).

$GABA_B$ receptors in the hippocampus (Davies et al., 1993). In cells transfected with either $GABA_{B1/2}$ or $GABA_{B2}$ subunits, the inhibition of Ca^{2+} current by 5 μM baclofen was prevented by exposure of the same cells to 1 μM CGP 55845A (Fig. 3A). These effects were highly significant ($GABA_{B1/2}$, $P<0.005$; $GABA_{B2}$, $P<0.0005$, paired t-tests; Fig. 3B). Note that the current inhibition for each transfection condition in the absence of antagonist cannot be compared (see legend to Fig. 3).

The inhibition of neuronal Ca^{2+} current by metabotropic receptor activation often has a substantial voltage-dependent component, which involves direct G protein signalling between receptor and channel (Zamponi and Snutch, 1998). The contribution of voltage-dependent inhibition can be demonstrated by the reduced effect of receptor activation when a large depolarising prepulse precedes the test voltage pulse. We assessed this phenomenon for transfections of $GABA_{B1/2}$ and $GABA_{B2}$ subunits. Fig. 4 shows, for a $GABA_{B2}$ subunit-transfected cell, that the inhibition of Ca^{2+} current by baclofen following a prepulse to +120 mV is substantially

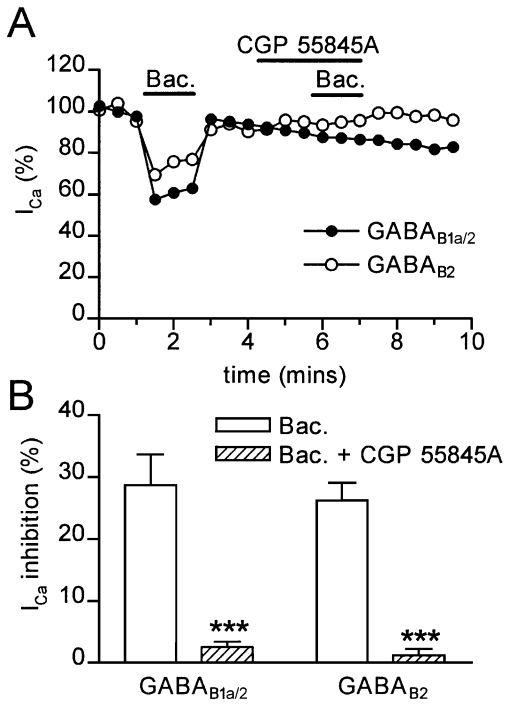

Fig. 3. A selective $GABA_B$ antagonist prevents current modulation in cells transfected with $GABA_{B1/2}$ and $GABA_{B2}$ subunits. (A) Plot of percentage Ca^{2+} current (I_{Ca}) is shown for two cells under the indicated transfection conditions. The bars indicate the periods of exposure to 5 μM baclofen (Bac.; ×2) and the antagonist, CGP55845A at 1 μM. (B) Percentage inhibition of Ca^{2+} current by baclofen (I_{Ca} inhibition) in the presence and absence of CGP55845A for transfections of $GABA_{B1/2}$ and $GABA_{B2}$ subunits ($n=5$, for both conditions). For these data, the mean response to baclofen in cells transfected with $GABA_{B2}$ subunits is much higher than shown previously (Fig. 1). This is because cells that exhibited robust responses were selected in order to emphasise the effect of the antagonist. No such selection of data took place for the results shown in Fig. 1, which is a separate data set.

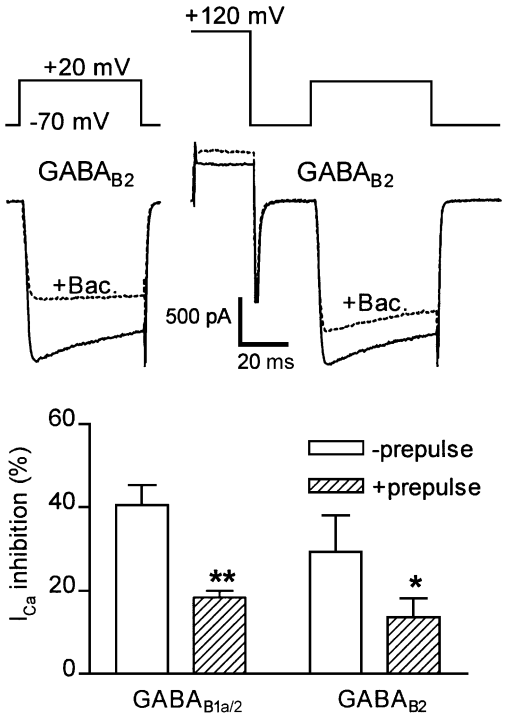

Fig. 4. A voltage prepulse partially reverses the inhibition of Ca^{2+} current by baclofen. The top traces show the voltage protocol and below these are examples of current traces obtained in the absence and presence of baclofen (50 μM). The experimental procedure is as follows. All recordings were made in nifedipine (10 μM). In the absence of baclofen, the current is recorded during two voltage steps to +20 mV. A 25 ms pulse to +120 mV precedes the second step. Immediately after this, baclofen perfusion is commenced, and 30 s later, the same protocol is applied. The traces illustrated were obtained from a cell transfected with $GABA_{B2}$ plasmid. The graph plots the mean data for the percentage inhibition of Ca^{2+} current (I_{Ca} inhibition) without and with the prepulse for transfections of $GABA_{B1/2}$ ($n=4$) and $GABA_{B2}$ ($n=3$) subunits. For $GABA_{B2}$ subunit transfections, only cells that exhibited a robust response to baclofen were used and therefore current inhibition is higher than seen in Fig. 1.

smaller than that without the prepulse. The mean data shows that the reduction in inhibition caused by the prepulse was similar for each transfection condition (Fig. 4). This effect was significant for cells transfected with $GABA_{B1/2}$ and $GABA_{B2}$ subunits ($P<0.005$ and $P<0.05$, respectively; paired t-tests).

The similarities in the functional responses achieved following $GABA_{B1/2}$ and $GABA_{B2}$ subunit transfections suggest either that the $GABA_{B1}$ subunit is not required to form functional receptors or that the NG108-15 cells express $GABA_{B1}$ receptor subunits endogenously and that functional heteromeric receptors are formed when recombinant $GABA_{B2}$ subunit is available. We used an antisense strategy to pursue this. Thus, antisense oligodeoxynucleotides targeted to the $GABA_{B1}$ subunit sequence close to the N-terminus were included in the transfection medium, which also contained the $GABA_{B2}$ subunit plasmid. Two different antisense oligonucleotides (ASI; ASII; see Materials and methods for sequences) directed against nearby regions of the $GABA_{B1}$ subunit were used together with corresponding missense oligonucleotides (MSI and MSII), which acted as controls. Fig. 5A shows data from two cells indicating that the baclofen inhibition of Ca^{2+} current is abolished by ASI treatment, but not by MSI. Mean results for both ASI and

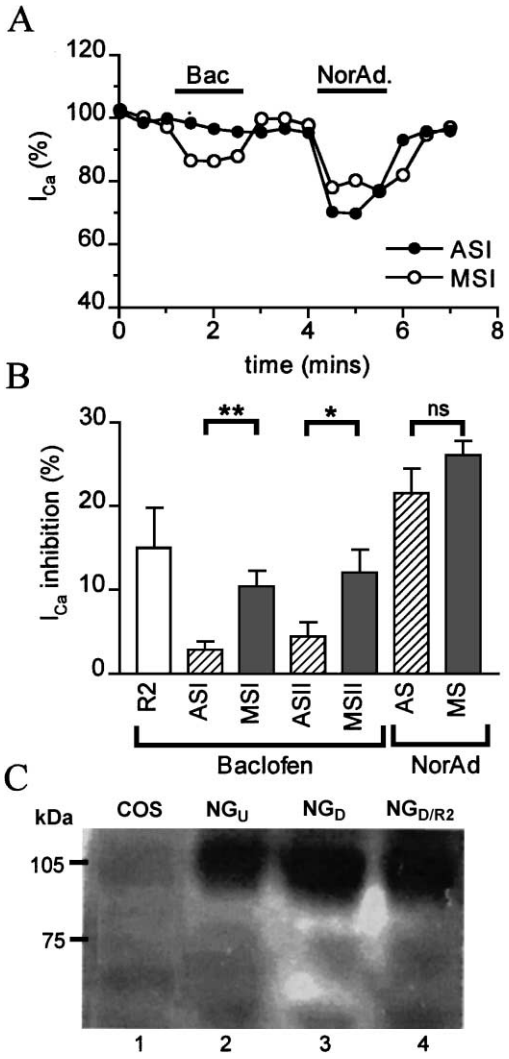

Fig. 5. NG108-15 cells contain endogenous $GABA_{B1}$ subunit. (A) A $GABA_{B1}$ subunit antisense oligonucleotide abrogates the effect of baclofen but not noradrenaline (NorAd). The percentage change in Ca^{2+} current (I_{Ca}) is plotted for two cells. The cells were transfected with $GABA_{B2}$ subunit plasmid and either an antisense or a missense oligonucleotide (ASI or MSI; see Materials and methods). The bars indicate the periods of exposure to baclofen (Bac; 50 μM) and noradrenaline (NorAd; 10 μM). (B) Mean percentage inhibition of Ca^{2+} current (I_{Ca} inhibition) by baclofen for ASI ($n=6$), MSI ($n=14$), ASII ($n=11$) and MSII ($n=12$). The effect of noradrenaline (NAdr) is illustrated on combined data from the antisense ($n=19$) and missense ($n=26$) treatments. For all conditions, cells were transfected with $GABA_{B2}$ subunit plasmid and the open bar shows the data for cells that were not treated with oligonucleotide ($n=23$, reproduced from Fig. 1C). Statistical comparisons were made between the indicated data sets (see text). (C) Western immunoblotting shows the presence of $GABA_{B1}$ subunit protein in NG108-15 cells. Cell extracts were immunoblotted with $GABA_{B1a}$ subunit antibodies. Lane 1, extracts from COS cells. Lane 2, undifferentiated NG108-15 cells. Lane 3, differentiated NG108-15 cells. Lane 4, differentiated NG108-15 cells transfected with $GABA_{B2}$ subunit plasmid. Molecular weight markers are shown on the left.

ASII confirm that these treatments significantly reduce Ca^{2+} current modulation compared to MSI and MSII, respectively (Fig. 5B; ASI vs. MSI, $P<0.005$; ASII vs. MSII, $P<0.05$, *t*-test). Further support for the specificity of the antisense effect was provided using noradrenaline, which produced very similar inhibition of Ca^{2+} current in antisense- and missense-treated cells (Fig. 5A,B; $P>0.05$, *t*-test). These data include two cells treated with antisense that exhibited minimal responses to noradrenaline (and baclofen). However, they were excluded from the analysis of baclofen's action to avoid any bias unrelated to antisense. Therefore, these results suggest that recombinant $GABA_{B2}$ and endogenous $GABA_{B1}$ subunits can associate to form functional receptors. We wished to confirm, however, that $GABA_{B1}$ receptor protein is present in NG108-15 cells and also to find out whether $GABA_{B2}$ subunits need to be expressed before the $GABA_{B1}$ subunit appears. Therefore, we carried out Western blotting of membranes from NG108-15 cells as well as COS cells using an antibody selective for the $GABA_{B1a}$ subunit (Fig. 5C). These data are not conclusive but they suggest that $GABA_{B1}$ subunit protein is present in NG108-15 cells, even in the untransfected and undifferentiated states, and does not appear to be expressed in COS cells.

In the final set of experiments, the voltage-gated Ca^{2+} channel subtypes inhibited by baclofen in cells transfected with $GABA_{B1/2}$ subunits were identified. Differentiated NG108-15 cells contain a variety of Ca^{2+} channel subtypes (Lukyanetz, 1998) and our experiments confirm the presence of at least L- and N-type channels. Current mediated by L-type channels was measured directly using nifedipine (Fig. 6A) and accounted for $25\pm5\%$ ($n=4$) of total current. From experiments where L-type channels were preblocked (Fig. 6C; $n=3$), ω-conotoxin GVIA was used to determine the amount of current carried via N-type channels, which was calculated to be 59% (see Materials and methods).

The effects of baclofen on Ca^{2+} channel subtypes were determined by measuring current density inhibition in the presence of channel blocking agents (see Materials and methods). The results from individual cells show that: (1) In the presence of the L-type Ca^{2+} channel blocker, nifedi-

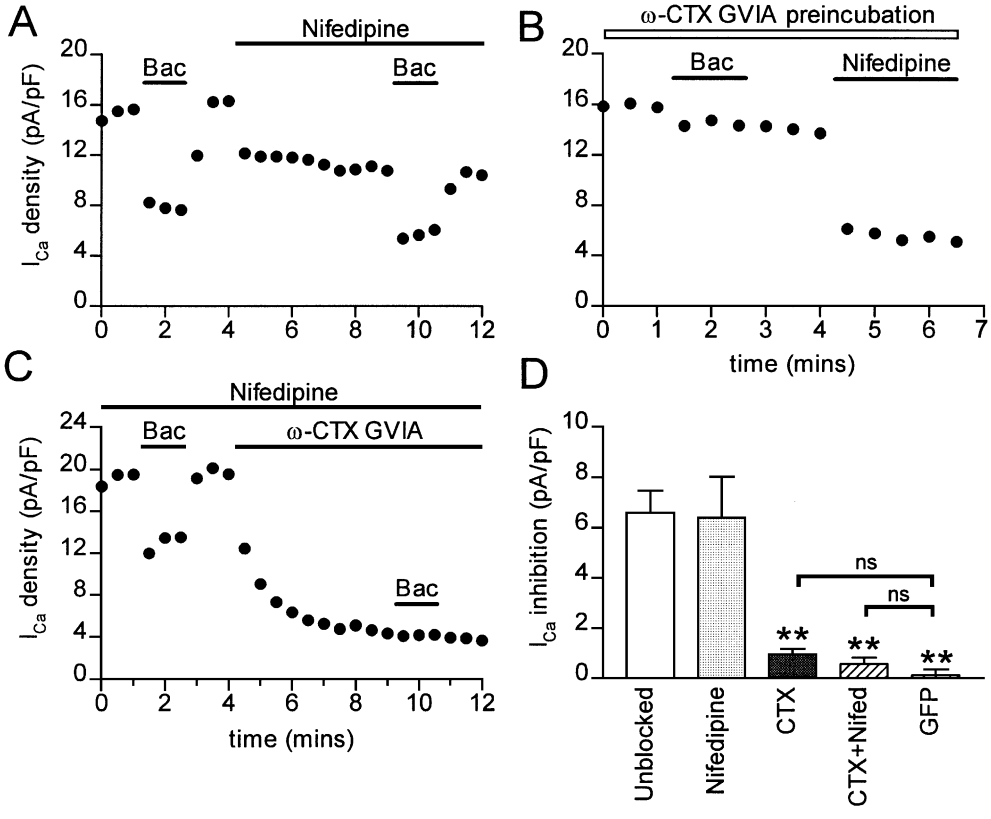

Fig. 6. $GABA_B$ receptors couple to N-type Ca^{2+} channels. (A–C) Plots of Ca^{2+} current density (I_{Ca} density) for individual cells showing the effects of Ca^{2+} channel blockers upon baclofen's action. Cells were transfected with $GABA_{B1a}$ and $GABA_{B2}$ subunits. The solid bars indicate drug application periods and the following concentrations were used: baclofen (Bac), 50 μM; nifedipine, 10 μM; ω-conotoxin (CTX)-GVIA, 1 μM. Specific features of the protocols are as follows. In A, no channel blocker was applied to the cell prior to establishing the recording. In B, the open bar indicates that the culture dish was incubated in ω-conotoxin-GVIA for 15 min and then washed out prior to making the recording. Some cells were left with a very small current under this condition but these were excluded from the analysis to emphasise the minimal effect of baclofen upon non-N-type current (though this may actually overestimate the effect of baclofen). Note that these recordings were not used to estimate the proportion of current subtypes (see Materials and methods). In C, nifedipine was applied to the cell at all times during the recording. (D) Mean current density inhibition caused by baclofen (I_{Ca} inhibition) for unblocked current ($n=8$) and current blocked by nifedipine ($n=7$), ω-CTX-GVIA ($n=6$) and ω-CTX-GVIA with nifedipine ($n=3$). The bar labelled GFP refers to cells that were transfected only with plasmid encoding green fluorescent protein and not exposed to channel blocking drugs ($n=25$).

pine, baclofen still brought about substantial current density inhibition (Fig. 6A). (2) The amount of current inhibition by baclofen was very small after block of current mediated by N-type channels (cells were preincubated in ω-conotoxin GVIA; Fig. 6B). The subsequent application of nifedipine showed that L-type channels contributed much of the remaining current. (3) When nifedipine was present throughout the recording period, the effect of baclofen was mostly removed only in the additional presence of ω-conotoxin GVIA (Fig. 6C). These findings were confirmed by analysing the mean data, which were drawn from the various experimental protocols, using an ANOVA followed by Tukey's test. Thus, in the absence of blockers, the mean reduction in current density induced by baclofen was not significantly different from that in the presence of nifedipine ($P>0.05$). However, the effect of baclofen is highly significantly reduced by ω-conotoxin GVIA preincubation ($P<0.001$) as well as by the combined application of ω-conotoxin GVIA and nifedipine ($P<0.001$).

Although the above data indicate that the effect of baclofen is transduced predominantly via N-type channels, we also wished to assess whether the effect of baclofen in the presence of ω-conotoxin GVIA was itself significantly different from background. Therefore, we made comparisons of these data with cells not transfected with $GABA_B$ receptor subunits and in which blockers were not applied (Fig. 6D). However, the current density change elicited by exposure to baclofen in cells transfected only with the plasmid encoding green fluorescent protein was not significantly different from that observed in transfections of $GABA_{B1/2}$ subunits in the combined presence of nifedipine and ω-conotoxin GVIA ($P>0.05$) or even in ω-conotoxin GVIA alone ($P>0.05$).

4. Discussion

$GABA_B$ receptor-mediated inhibition of Ca^{2+} current is evident in differentiated NG108-15 cells following polyethylenimine-induced transfection of plasmids encoding $GABA_{B1a}$ and $GABA_{B2}$ subunits. In a previous study, we used electroporation to achieve $GABA_B$ subunit plasmid transfections but the response to baclofen in fluorescent (green fluorescent protein-expressing) cells was quite small, especially for single subunit transfections, and it was difficult to make any in-depth interpretation of these data (Easter and Spruce, 2000). Although voltage-gated Ca^{2+} channel modulation is a crucial function of native $GABA_B$ receptors, only one other group has demonstrated this activity for the recombinant receptors (Filippov et al., 2000). Differentiated NG108-15 cells that have not been transfected with $GABA_B$ subunit plasmids exhibit an insignificant response to baclofen, despite the fact that they are known to contain a variety of G-protein-coupled neurotransmitter receptors, some of which have been shown to couple to voltage-gated Ca^{2+} channels (Brown et al., 1989). Though we have not demonstrated the direct involvement of G proteins, there is abundant evidence that the recombinant $GABA_B$ receptors mediate their effects via G protein activation (Couve et al., 2000).

Ca^{2+} current modulation is also observed when cells are transfected with the $GABA_{B2}$ subunit alone, though to a lesser extent than for the double transfections. This might suggest the formation of functional homomeric receptors, as has been proposed previously (Martin et al., 1999). Moreover, for transfections of $GABA_{B1/2}$ and $GABA_{B2}$ subunits, a selective $GABA_B$ receptor antagonist is equally effective and the voltage dependence of current modulation is very similar, indicating that the receptor make-up and coupling mechanism are the same whether or not $GABA_{B1}$ subunit is included in the transfection protocol. However, there is very good evidence from a variety of other studies that infer that the functional receptor in NG108-15 cells contains both $GABA_{B1}$ and $GABA_{B2}$ subunits, even when only the latter subunit is transfected. Thus, ligands have been shown to bind only to the $GABA_{B1}$ subunit (Malitschek et al., 1999; Galvez et al., 2000). In addition, in native neuronal tissues, $GABA_B$ receptor function is absent when $GABA_{B1}$ subunit expression is disrupted (Hand et al., 2000; Prosser et al., 2001). The $GABA_{B1}$ subunit is not itself sufficient to form functional receptors, however, as many studies demonstrate (reviewed in Bowery and Enna, 2000), including the present results. Finally, the role for the $GABA_{B2}$ subunit has been defined. It has been established that, in isolation, the $GABA_{B1}$ subunit is retained in the endoplasmic reticulum (Couve et al., 1998) and that the $GABA_{B2}$ subunit is needed to transport $GABA_{B1}$ into the surface membrane (reviewed by Couve et al., 2000). Moreover, the $GABA_{B2}$ subunit has an important role in the receptor complex, for activating signal transduction pathways (Galvez et al., 2001). Therefore, in the current study, the results from single subunit transfections in NG108-15 cells are most likely explained by the presence of endogenous $GABA_{B1}$ subunit, which can form functional receptors only when recombinant $GABA_{B2}$ is available. Evidence for this is provided using $GABA_{B1}$ subunit antisense oligonucleotides, which disrupt current modulation in $GABA_{B2}$ subunit-transfected cells, and by demonstrating $GABA_{B1}$ subunit protein in NG108-15 cells using Western blotting. Selectivity of the antisense oligonucleotides is indicated by a number of findings: similar results for two different antisense oligonucleotides; significantly greater modulation using corresponding missense oligonucleotides; and the lack of disruption of the current modulation induced by noradrenaline by any type of oligonucleotide.

We also investigated the Ca^{2+} channel subtypes that are modulated by the recombinant receptors. Only cells transfected with $GABA_{B1/2}$ subunits were used. Differentiated NG108-15 cells are known to express a variety of Ca^{2+} channel subtypes, including N-, T-, L- and possibly Q-types (Lukyanetz, 1998), though we used a membrane potential at which the contribution of T-type current would be reduced. The cells appeared to have appropriate proportions of at

least the L- and N-channel types. Our data indicates that the recombinant receptors couple only to N-type channels since no significant current modulation was observed when this channel type was blocked. We assumed that we achieved selective block of N-type channels by preincubation of cells in ω-conotoxin GVIA followed by washout of this toxin. This method was used to avoid additional block of L-type channels by this toxin (Seabrook et al., 1994; Connor and Henderson, 1997). Indeed, a majority of the residual current was blocked by nifedipine. Modulation of N-type channels by baclofen is a common finding in native neuronal tissues (Scholz and Miller, 1991; Menon-Johansson et al., 1993; Amico et al., 1995; Guyon and Leresche, 1995; Sun and Chiu, 1999). Coupling of $GABA_B$ receptors to other Ca^{2+} channel types has also been described, but for L-type channels there is inconsistency, which may be ascribed to the neuronal type, as to whether this subtype can be modulated (Amico et al., 1995; Scholz and Miller, 1991) or not (Mintz and Bean, 1993; Doze et al., 1995). Nevertheless, it is surprising that, in NG108-15 cells, modulation of L-type current is not detected since, in heterologous expression systems, promiscuous coupling of metabotropic receptors to second messenger pathways is often seen. One explanation for this may arise from the finding that neurones may contain subtypes of L-type channels that are differentially sensitive to modulation by metabotropic receptor activation (Amico et al., 1995). In fact, brain tissue is known to express subtypes of L-type channels (Dolphin, 1995). Therefore, if only, at most, a proportion of the L-type Ca^{2+} current present in NG108-15 cells is susceptible to modulation, we may not 2+ channels presumed to occur via binding of G protein subunits. More subtle effects of baclofen on current properties have not been assessed in this study. It is possible that changes in current kinetics and voltage dependence of inactivation of the non-N current subtypes may have resulted from the activation of the recombinant $GABA_B$ receptors.

To summarise, we have shown that recombinant $GABA_B$ receptors couple to N-type Ca^{2+} channels in differentiated NG108-15 cells. These cells contain endogenous $GABA_{B1}$ subunit that is able to form functional receptors when recombinant $GABA_{B2}$ subunit is available.

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