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Analysis of $GABA_A$- and $GABA_B$-receptor mediated effects on intracellular Ca^{2+} in DRG hybrid neurones

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1 Using pharmacological analysis and fura-2 spectrofluorimetry, we examined the effects of γ-aminobutyric acid (GABA) and related substances on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of hybrid neurones, called MD3 cells. The cell line was produced by fusion between a mouse neuroblastoma cell and a mouse dorsal root ganglion (DRG) neurone.

2 MD3 cells exhibited DRG neurone-like properties, such as immunoreactivity to microtubule-associated protein-2 and neurofilament proteins. Bath applications of capsaicin and α, β-methylene adenosine triphosphate reversibly increased $[Ca^{2+}]_i$. However, repeated applications of capsaicin were much less effective.

3 Pressure applications of GABA (100 μM), (Z)-3-[(aminoiminomethyl) thio] prop-2-enoic acid sulphate (ZAPA; 100 μM), an agonist at low affinity $GABA_A$-receptors, or KCl (25 mM), transiently increased $[Ca^{2+}]_i$.

4 Bath application of bicuculline (100 nM – 100 μM), but not picrotoxinin (10 – 25 μM), antagonized GABA-induced increases in $[Ca^{2+}]_i$ in a concentration-dependent manner ($IC_{50} = 9.3$ μM).

5 Ca^{2+}-free perfusion reversibly abolished GABA-evoked increases in $[Ca^{2+}]_i$. Nifedipine and nimodipine eliminated GABA-evoked increases in $[Ca^{2+}]_i$. These results imply GABA response dependence on extracellular Ca^{2+}.

6 Baclofen (500 nM – 100 μM) activation of $GABA_B$-receptors reversibly attenuated KCl-induced increases in $[Ca^{2+}]_i$ in a concentration-dependent manner ($EC_{50} = 1.8$ μM). 2-hydroxy-saclofen (1 – 20 μM) antagonized the baclofen-depression of the KCl-induced increase in $[Ca^{2+}]_i$.

7 In conclusion, $GABA_A$-receptor activation had effects similar to depolarization by high external K^+, initiating Ca^{2+} influx through high voltage-activated channels, thereby transiently elevating $[Ca^{2+}]_i$. $GABA_B$-receptor activation reduced Ca^{2+} influx evoked by depolarization, possibly at Ca^{2+}-channel sites in MD3 cells.

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Abbreviations: α,β-CH_2-ATP, α,β-methylene adenosine triphosphate; $[Ca^{2+}]_i$, Intracellular free calcium concentration; DMEM, Dulbecco's modified Eagle's medium; DNase, Deoxyribonuclease; DRG, Dorsal root ganglion; EC, Effective concentration (e.g., 50% of maximum); EGTA, Ethylene glycol-bis(β-aminoethylether)N,N,N′,N′ tetraacetic acid; E_{GABA}, Reversal potential for GABA action; FBS, Foetal bovine serum; GABA, γ-Aminobutyric acid; HHBSS, HEPES-buffered Hank's balanced salt solution; HPRT, Hypoxanthine phosphoribosyl transferase; HS, Horse serum; IC, Inhibitory concentration (e.g., 50% of maximum); K_D, Dissociation constant; MAP-2, Microtubule-associated protein-2; MOPS, (3-[N-morpholino]propanesulphonic acid); NF-H, High molecular weight neurofilament protein; ZAPA, ((Z)-3-[9-aminoiminomethyl)thio]prop-2-enoic acid sulphate

Introduction

γ-Aminobutyric acid (GABA) and related agonists have well-established effects on dorsal root ganglion (DRG) neurones that convey information from peripheral sensory receptors to the central nervous system (CNS). Application of GABA to the DRG activates $GABA_A$ receptors, increasing membrane Cl^- conductance (Alvarez-Leefmans *et al*., 1998). The selective $GABA_B$ agonist, baclofen, activates metabotropic $GABA_B$ receptors, affecting K^+ and T-type Ca^{2+} currents and inhibiting high voltage-activated Ca^{2+} currents (Robertson & Taylor, 1986; Bowery, 1993). When Cl^- and K^+ currents are minimized, GABA application reduces voltage-activated Ca^{2+} currents (Deisz & Lux, 1985). The application of GABA or baclofen shortens the DRG action potential duration by decreasing an N-type Ca^{2+}-current (Dunlap & Fischbach, 1981; Green & Cottrell, 1988). However, acutely dissociated human DRG neurones do not exhibit responses to baclofen or saclofen, a $GABA_B$ antagonist (Valeyev *et al*., 1996).

The effects of GABA on DRG somata are intriguing because of a scarcity of synapses in the DRG (Kayahara, 1986) and the

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observations that action potentials travelling along the peripheral afferent fibres can bypass the somata on the way to the spinal cord (Tagini & Camino, 1973). However, axonal stimulation produces action potential invasion of primary afferent cell bodies (Ito, 1957) and these neurones can display spontaneous action potentials under various conditions (Kirk, 1974; Amir & Devor, 1996). There are demonstrations of chemical communication between neighbouring neurones in the DRG (Amir & Devor, 1996) and intraganglionic release of substance P (Neubert *et al*., 2000).

These observations on DRG somata are likely important for explaining how intrathecal drug application produces therapeutic effects in patients with pain disorders (Gordon *et al*., 1995; van Hilten *et al*., 2000). Hence, despite the physiological uncertainties, understanding the receptor systems for GABA and other modulators in the DRG is critical for the development of drugs affecting sensory systems.

In the studies reported here, we have examined the GABA responses of hybrid neurones derived from DRG neurones and neuroblastoma cells. Immortalized hybrid sensory neurones will be valuable models for pharmacological research and drug discovery. A renewable source of relatively homogeneous neurones greatly facilitates the clarification of drug-receptor mechanisms and the development of high-throughput screens for drug candidates. When used with recombinant techniques and manipulated receptor expression, the approach can lead to discovery of novel drug targets for the treatment of pain and other disorders. A first step is the elucidation of the responses in a sensory neurone cell line, possessing native receptors. Hence, we have investigated, for the first time, the effects of GABA on the intracellular free calcium concentration ($[Ca^{2+}]_i$) of this mouse hybrid DRG cell line. Furthermore, we have assessed the effects of agonists, antagonists and ion channel blockers and clarified the involvement of voltage-activated Ca^{2+} channels in the actions of GABA on these neurones.

Methods

Cell Culture

A hybrid cell line was derived by somatic cell fusion of a hypoxanthine phosphoribosyl transferase (HPRT)-deficient neuroblastoma cell line N18TG2 with DRG neurones from Balb/C mice (Hammond *et al*., 1986). In brief, DRG neurones were removed from 3-week-old Balb/C mice and incubated for 2 h at 37°C in phosphate-buffered saline (PBS, Dulbecco 'A'), containing 0.25% collagenase (Worthington Biochemical, Freehold, NJ, U.S.A) and 20 μg ml^{-1} DNase (Sigma, St. Louis, MO, U.S.A), and then dissociated into single cells by repeated pipetting. Dissociated DRG neurones were re-suspended in a feeding medium consisting of Dulbecco's modified Eagle's medium (DMEM), 5% foetal bovine serum (FBS), 5% horse serum (HS) and 20 μg ml^{-1} gentamicin and plated in six well plates, previously coated with 10 μg ml^{-1} poly-D-lysine. Two days later, the DRG cultures were exposed to 10 μM fluorodeoxyuridine and 10 μM uridine for the subsequent 24 h in order to inhibit the growth of Schwann cells and fibroblasts, enriching the cultures with DRG neurones. Seven to 10 days later, N18TG2 mouse neuroblastoma cells were added to the DRG neuronal cultures along with the feeding medium containing 40 μg ml^{-1} phytohemagglutinin which facilitated the adherence of the neurones to the N18TG2 cells. After 15 min of incubation, the medium was aspirated and the cells were fused with 50% (v v^{-1}) polyethylene glycol (4000, Sigma). The fusion products were plated in DMEM containing 5% FBS, 5% HS, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) which discriminates against HPRT-deficient cells. After 7 days of treatment in HAT medium, the cultures were fed DMEM containing 5% FBS and 5% HS. The media were changed every 2–3 days. The colonies were recognized in the third week of culture, isolated and expanded further in DMEM containing 5% FBS, 5% HS, 20 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} fungizone. One expanded colony, named MD3, was used in the present study.

Immunocytochemistry

For immunocytochemical studies, MD3 cells grown on Aclar plastic coverslips (9 mm round) were cultured for 2–4 days, rinsed in PBS and fixed in methanol at −20 °C for 10 min. Cells were incubated with mouse monoclonal antibodies (Ab) against microtubule-associated protein-2 (MAP-2, 1 : 100; Sigma) or rat monoclonal antibodies against high molecular weight neurofilament protein (NF-H; 1 : 10, clone Ta51; gift of Dr V. Lee) at 4°C for 48 h. Then, the cells were washed three times in PBS and incubated in biotinylated anti-mouse or anti-rat IgG Ab (1 : 200) for 1 h at room temperature (~22°C) followed by another wash and incubation in ABC solution (Vector Laboratories, Mississauga, ON, Canada) using diaminobenzadine as a chromogen. The coverslips were dehydrated in ethanol, cleaned in xylene, and attached to microscopic slides with Permount.

Measurement of $[Ca^{2+}]_i$

Before measuring $[Ca^{2+}]_i$, MD3 cells were grown on poly-D-lysine-coated 22 mm glass coverslips (#1 thickness, Carolina Biological, Burlington, NC, U.S.A) for at least 12 h. The buffer solution was a HEPES-buffered Hanks' balanced salt solution (HHBSS, pH 7.4) containing (in mM) NaCl 145; KCl 2.5; $MgCl_2$ 1.0; HEPES 20; glucose 10; and $CaCl_2$ 1.8. To ensure that the Ca^{2+}-free media had no residual Ca^{2+} in the buffer solution, 50 μM EGTA was substituted for 1.8 mM Ca^{2+} in HHBSS. The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator, fura-2-acetoxymethylester (fura-2; Molecular Probes, Eugene, OR, U.S.A), as described previously (Yoo *et al*., 1999). The cells were incubated in fura-2 (5 μM), dissolved in dimethylsulphoxide and further solubilized with pluronic acid (0.2%) in HHBSS containing 0.02% bovine serum albumin at 37°C for 45 min. Then, the cells were washed twice in HHBSS and incubated for an additional 15 min to ensure complete de-esterification of fura-2. Coverslips with fura-2-loaded cells were mounted in a perfusion chamber (working volume, 0.4 ml) and fitted into the stage of an inverted microscope.

Fluorescence signals of fura-2 were measured using a Diaphot TMD inverted microscope (Nikon, Tokyo, Japan), equipped with a Nikon fluorite epifluorescence objective (40×) and a Xenon light source (100 W). Emitted fluorescence was detected by a silicone intensified target video

camera (Hamamatsu C2400-08), then digitized by an analogue-to-digital video interface and observed with image-detection software (Image-1/FL, Universal Imaging, West Chester, PA, U.S.A.) running on an IBM-compatible computer and stored on hard disc. Fura-2 was excited at 340 nm and 380 nm wavelengths. The excitation-ratios were obtained from 8-frame averages of pixel intensities at each excitation frequency. The results were transferred to a data spreadsheet where the ratios were converted to the actual calcium concentrations (Grynkiewicz *et al*., 1985). Cells for $[Ca^{2+}]_i$ measurements were selected on the basis of approximately spherical cell morphology, appreciable fluorescence, indicating an adequate cytoplasmic uptake of fura-2, and a resting $[Ca^{2+}]_i$ that was stable for a >5 min period. In this selection, we avoided clumps which comprised a group of adherent cells. Usually, we selected 5 cells for $[Ca^{2+}]_i$ measurement per coverslip or experiment.

The imaging system was calibrated using a pentapotassium salt of fura-2 (5 μM, Molecular Probes, Eugene, OR, U.S.A.) and a series of buffers containing 100 mM KCl, 10 mM MOPS and ratios of Ca^{2+}-EGTA to EGTA that yielded Ca^{2+} concentrations between 0 and 39.8 μM (pH 7.2, Molecular Probes) at 25°C. The dissociation constant (K_D) for the fura-2 imaging system under these experimental conditions was 221 nM (*cf*. Grynkiewicz *et al*., 1985).

Drugs

The following stock solutions were prepared in HHBSS: GABA, nifedipine, nimodipine, α,β-methylene adenosine triphosphate (α,β-CH_2-ATP), 8-methyl-N-vanillyl-6-nonenamide (capsaicin), diazepam (all from Sigma), (Z)-3-[(aminoiminomethyl) thio] prop-2-enoic acid sulphate (ZAPA; Tocris Cookson, Ballwin, MO, U.S.A.), bicuculline methobromide, (±) baclofen, picrotoxinin and 2-hydroxy-saclofen (all from Precision Biochemicals, Vancouver, BC, Canada). The stock solutions of capsaicin, diazepam, nimodipine and nifedipine, stored in 0.5–2 ml aliquots at −22°C, were initially dissolved in a small quantity of absolute ethanol and then diluted in HHBSS to a final ethanol concentration of $<0.01\%$.

Drug administration

Drugs were applied in the bath and by pressure-ejection from pipettes of 10–20 μm tip-diameters (Picospritzer, General Valve, Fairfield, NJ, U.S.A.). At the start of an experiment, GABA agonists or KCl were applied by pressure to initiate a response. If no response resulted, then either the ejection time was increased or the pipette tip was moved closer to the cells, using the micromanipulator. Care was taken to optimize the pipette tip-to-cell distance, such that a maximal response was obtained at a given pressure. The final pipette tip position usually was <100 μm (range, 50 to 200 μm) from the cells under study.

Statistical analyses

Statistical analyses were carried out with the use of Student's *t*-tests for comparison of two groups and for testing differences from a theoretical mean. In cases where recurrent agonist or KCl applications by pressure pulse resulted in Ca^{2+} responses, the peak amplitude of the first two responses (in some cases, three consecutive responses) were averaged and used as the average or control amplitude. At least three experiments were performed for each drug condition and the results are expressed as mean±s.e.mean, unless stated otherwise. Results were considered statistically significant when $P<0.05$. The data were analysed using Excel, GraphPad Prism, and StatView software.

Results

The purpose of the initial experiments was to determine similarities and dissimilarities of the hybrid MD3 cells to DRG neurones. Immunocytochemical labelling with monoclonal antibodies showed that MD3 cells expressed NF-H and MAP-2 proteins, both specific markers for neuronal phenotypes. The cell body diameters ranged between 10 and 25 μm and ~90% of the cells had two or more, short- or medium-sized processes. We then examined their responsiveness to bath applications of capsaicin which acts selectively on sensory neurones (Bevan & Szolcsanyi, 1990) and α,β-methylene adenosine-triphosphate, an agonist at purinergic receptors (Bouvier *et al*., 1991).

Responses to capsaicin and α,β-methylene adenosine-triphosphate

Bath application of 3 μM capsaicin transiently increased $[Ca^{2+}]_i$ to a peak of 295±21 nM in 47 out of 93 cells. The resting $[Ca^{2+}]_i$ averaged 89.6±7.4 nM ($n=28$). In all 47 responding cells, repeated applications of capsaicin did not evoke large increases in $[Ca^{2+}]_i$ after the first response, despite periods of up to 30 min between the applications. Two or three repeated applications of capsaicin (3 μM) evoked much smaller increases in $[Ca^{2+}]_i$, often ~70 % less than the first response, presumably due to an acute desensitization with a rapid onset. In five additional experiments, we pretreated the cells for 10 min with capsazepine (5 μM), a capsaicin-receptor antagonist, and subsequently observed little or no increase in $[Ca^{2+}]_i$ on first application of capsaicin.

To facilitate a rapid recovery and circumvent tachyphylaxis, we applied capsaicin (30 μM) by pressure from a micropipette. The applications transiently increased $[Ca^{2+}]_i$ to response amplitudes that had a mean peak value of 404±56 nM that lasted from 1 to 3 min in 14 out of 37 cells. In general, these responses were similar to observations in sensory neurones (Bevan & Szolcsanyi, 1990).

Bath applications of α,β-methylene adenosine-triphosphate (α,β-CH_2-ATP; 100 μM) for 10 s transiently increased $[Ca^{2+}]_i$ to an average peak value of 317±20 nM in 58 out of 63 cells. The $[Ca^{2+}]_i$ then returned to resting values within 4 to 6 min. In view of the known, rapid desensitization of such responses (Petruska *et al*., 2000), we applied 100 μM concentrations of α,β-CH_2-ATP by pressure from a micropipette. These applications transiently increased $[Ca^{2+}]_i$ to an average peak value of 262±18 nM in 11 out of 20 cells. These cells recovered within 1 min, i.e., much more rapidly than from bath applications. In four experiments, Ca^{2+}-free perfusion for 1 min reversibly eliminated the responses to bath applications of α,β-CH_2-ATP (100 μM for 10 s). Hence, the activation of receptors by α,β-CH_2-ATP in cells bathed in Ca^{2+}-containing media probably resulted in a Ca^{2+} influx.

Responses to GABA-agonist applications

We determined the effects of GABA on the $[Ca^{2+}]_i$ of MD3 cells. In preliminary experiments, we observed only small amplitude responses to bath applications of GABA, presumably due to an acute desensitization. We subsequently used brief applications of GABA, applied by pressure ejection from a pipette (containing 100 μM) close to the cells.

GABA increased $[Ca^{2+}]_i$ to an average peak value of 347 ± 42 nM ($n = 54$). Figure 1A shows typical responses consisting of reversible, transient elevations in $[Ca^{2+}]_i$ with little or no tachyphylaxis on repeated applications of GABA.

In 16 of 54 cells, however, application of GABA produced an increase in $[Ca^{2+}]_i$ that peaked and then declined to a plateau, before gradually decreasing to resting levels. The responses often had a triphasic pattern, consisting of an initially rapid rise to a peak $[Ca^{2+}]_i$ and a decline that formed a $\sim$40 s long plateau, followed by recovery at a much slower rate to the baseline.

Effects of thapsigargin and caffeine

We attempted to determine a possible involvement of internal Ca^{2+} sequestration in the GABA responses. For example, the slow decline or plateau may implicate sluggish Ca^{2+}-sequestration. We applied thapsigargin (1 – 10 μM in 15 cells) and caffeine (1 – 10 mM in 18 cells) in the bath for 5 min prior to local application of GABA. This pre-treatment with either thapsigargin or caffeine produced little or no change in the resting $[Ca^{2+}]_i$. We then observed little or no change in the amplitude of the GABA-induced increase in $[Ca^{2+}]_i$ during the co-application with either thapsigargin or caffeine (data not shown).

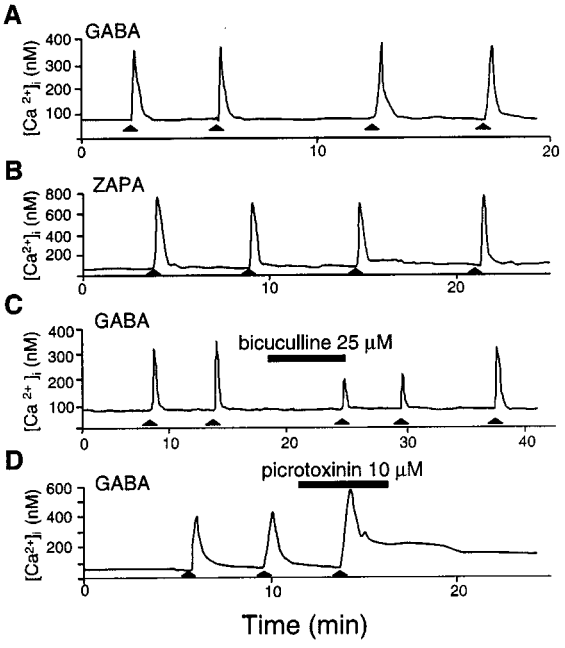

Figure 1 Effects of $GABA_A$-receptor activation on $[Ca^{2+}]_i$, demonstrated by applications of $GABA_A$-receptor agonists (indicated by arrowheads) and antagonists from a pipette positioned close to cells (A – D). (A) Repeated applications of GABA (100 μM) produced transient increases in $[Ca^{2+}]_i$. (B) Local applications of ZAPA (100 μM) transiently increased $[Ca^{2+}]_i$, similar to the GABA-evoked responses. (C) Bath application (bar, 5 min) of bicuculline (25 μM) reversibly blocked the GABA-evoked increases in $[Ca^{2+}]_i$. (D) Bath application (bar, 5.6 min) of picrotoxinin (10 μM) did not blunt the responses to GABA.

Effects of (Z)-3-[(aminoiminomethyl) thio] prop-2-enoate (ZAPA)

Local applications of ZAPA (100 μM), an agonist at low affinity $GABA_A$-receptors, increased $[Ca^{2+}]_i$ by 393 ± 44 nM in 24 cells out of 61 cells. ZAPA produced transient increases in $[Ca^{2+}]_i$ of consistent amplitude, usually declining without a pronounced plateau. Figure 1B shows the largest response obtained with ZAPA application. We did not observe a significant diminution in the response amplitude upon three to five consecutive applications of ZAPA. Since ZAPA does not significantly activate $GABA_B$- and $GABA_C$-receptors (Allan *et al.*, 1997), we assumed that $GABA_A$-receptors mediated the increase in $[Ca^{2+}]_i$ produced by GABA.

$GABA_A$-receptor involvement in the GABA-evoked responses

Effects of $GABA_A$-receptor antagonists We applied the $GABA_A$-receptor antagonists, bicuculline and picrotoxinin, in order to resolve the nature of the receptors responsible for mediating the response to GABA. During pre-treatment of cells with bicuculline (100 nM – 100 μM) or picrotoxinin (10 – 25 μM) for 3 – 6 min prior to co-application with GABA, there was no significant change in the resting $[Ca^{2+}]_i$. As shown in Figure 1C, bath application of bicuculline (100 nM – 100 μM) reversibly reduced the increases in $[Ca^{2+}]_i$ resulting from local applications of GABA ($n = 29$). Substantial recovery from this antagonism occurred within 10 to 15 min. The concentration-response curve for bicuculline showed an $IC_{50} = 9.3$ μM (Figure 2, *cf.* Table 1). On the other hand, bath application of picrotoxinin (10 – 25 μM) for 5 to 10 min did not decrease the $[Ca^{2+}]_i$ response evoked by GABA (100 μM) in 31 cells (Figure 1D). In nine of the 31 cells, picrotoxinin actually increased the responses to GABA by an average of $18 \pm 4\%$ over the control values. These cells exhibited little or no recovery to baseline values after picrotoxinin applications, despite observation periods of $\leqslant$20 min. However, the antagonism of the GABA-response by bicuculline implicated the involvement of $GABA_A$-receptors in the $[Ca^{2+}]_i$ response to GABA.

Effects of $GABA_B$-receptor antagonism Application of the $GABA_B$-receptor antagonist, 2-hydroxy-saclofen (20 μM), for 4 to 5 min did not affect the resting values of $[Ca^{2+}]_i$. In six experiments used for statistical analysis of the effects of this drug, we did not observe a significant change in the mean peak control responses to GABA (395 ± 78 nM), compared to the peak responses during combined application with 2-hydroxy-saclofen (406 ± 64 nM, $n = 37$).

Effects of Diazepam

We applied diazepam to determine if the benzodiazepine receptor is a functional part of $GABA_A$-receptors in MD3 cells, as described in CNS and DRG neurones (Akaike *et al.*, 1989). Bath applications of diazepam (1 – 50 μM) for 20 min

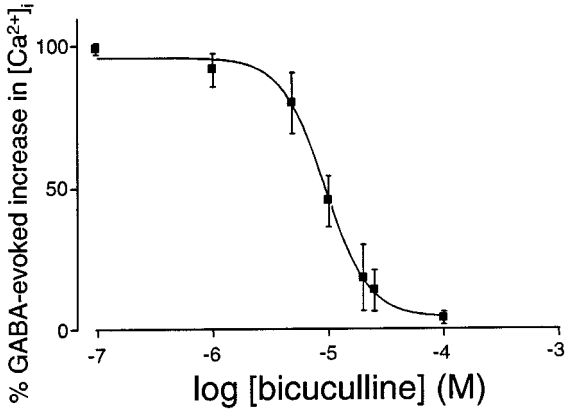

Figure 2 Concentration-response relationship for bicuculline-induced blockade of GABA-mediated increase in $[Ca^{2+}]_i$. Following two or more control responses to GABA (*cf.* Figure 1A), bath application of bicuculline (100 nM – 100 μM) blocked the GABA-evoked increase in $[Ca^{2+}]_i$. The inhibition was calculated as a percentage of the averaged controls. The IC_{50} for bicuculline was 9.3 μM. The relationship between the mean per cent inhibition and the logarithm of the bicuculline concentration was fitted with a sigmoidal function using GraphPad software. Data represent per cent GABA-evoked increase in $[Ca^{2+}]_i$ (mean ± s.e mean, Table 1).

Table 1 Effects of bicuculline on GABA-induced increase in $[Ca^{2+}]_i$

[Bicuculline] (μM)	*$[Ca^{2+}]_i$* (% of control)	P *value*	n
0.1	99.3 ± 7.0	n.s.	4
1	91.8 ± 5.9	n.s.	4
5	80.0 ± 10.8	n.s.	4
10	45.2 ± 8.9	< 0.001	5
20	18.3 ± 11.7	0.0001	5
25	13.6 ± 7.3	< 0.0001	3
100	3.8 ± 2.4	< 0.0001	4

Inhibition of GABA-evoked increases in $[Ca^{2+}]$i was measured at 5 min of bicuculline application (bath). Inhibition of the GABA-evoked response was calculated as a fraction of control values that were an average of two or more peak elevations in $[Ca^{2+}]$i due to local GABA (100 μM) applications. The values are expressed as per cent of control amplitude (mean ± s.e.mean; n.s. = non-significant). The *P* values indicate statistical differences from control values (Student's *t*-test). '*n*' refers to the number of experiments studied at a given concentration.

did not significantly affect the resting $[Ca^{2+}]_i$ or the responses evoked by local applications of GABA in five experiments (data not shown). The failure of diazepam applications to potentiate the GABA-stimulated response implies that some differences in $GABA_A$-receptors exist between MD3 cells and DRG neurones.

Mechanism of the GABA receptor-induced increase in $[Ca^{2+}]_i$

Effects of high extracellular $[K^+]$ We determined the ionic mechanisms subsequent to $GABA_A$-receptor activation that led to the increased $[Ca^{2+}]_i$. We investigated the possibility that a membrane depolarization evoked by GABA would facilitate Ca^{2+} entry through voltage-activated Ca^{2+} channels. We locally applied a high $[K^+]$ to induce a depolarization and compared the $[Ca^{2+}]_i$ responses to GABA applications. As shown in Figure 3A, an increase in $[K^+]$ from 2.5 – 25 mM transiently increased $[Ca^{2+}]_i$ to ~800 nM. In 58 cells, KCl application increased $[Ca^{2+}]_i$ by average of 538 ± 60 nM, mimicking the effects of GABA.

Effects of Ca^{2+}-removal from extracellular perfusion

During perfusion with Ca^{2+}-free media, the application of GABA (100 μM) or KCl (25 mM), did not produce a rise in $[Ca^{2+}]_i$ (Figure 3A,B). The Ca^{2+}-free perfusion, while sometimes slightly reducing the resting $[Ca^{2+}]_i$, completely and reversibly blocked the responses to applications of KCl ($n = 21$) and GABA ($n = 18$). After returning to Ca^{2+}-containing media, applications of GABA or KCl were again as effective in elevating $[Ca^{2+}]_i$ as in the previous controls. In view of the absence of GABA- or KCl-evoked effects during perfusion with nominally Ca^{2+}-free media, we considered the possibility that application of GABA and KCl raised $[Ca^{2+}]_i$ by activating voltage-dependent Ca^{2+}-currents.

Dihydropyridines inhibit GABA- and KCl-induced increase in $[Ca^{2+}]_i$

By applying Ca^{2+}-channel blockers, we assessed a contribution of voltage-gated Ca^{2+}-currents to the GABA-evoked elevation in $[Ca^{2+}]_i$ in 47 cells. In these experiments, we measured the effects of bath application (5 min) of nifedipine (1 – 20 μM), nimodipine (1 – 20 μM) or vehicle (0.01% alcohol) on the amplitude of the responses to local applications of GABA (100 μM) and KCl (25 mM). Application of the vehicle, alone, had no significant effect on the resting $[Ca^{2+}]_i$ or on GABA- and KCl-evoked responses in four cells. However, application of nifedipine or nimodipine attenuated both the KCl- and GABA-stimulated responses, reducing the amplitude by > 50%, and sometimes completely blocking the Ca^{2+} transients (Figure 3C,D). In a majority of cells, dihydropyridine application (10 or 20 μM) eliminated the responses to KCl and GABA. We observed full recovery from a dihydropyridine application in 25 out of 47 cells. Perfusion with normal media for 10 – 15 min only partly reversed the blockade produced by either nifedipine or nimodipine in the remaining 22 cells. These data implicate an influx of Ca^{2+} through voltage-activated Ca^{2+}-channels in the GABA- and KCl-evoked responses.

$GABA_B$-receptor involvement in the GABA-evoked response

We investigated the effects of baclofen, a $GABA_B$-receptor agonist, on MD3 cells. Baclofen application (50 nM to 100 μM) for 3 – 5 min produced no significant effects on the resting $[Ca^{2+}]_i$ ($n = 87$). Baclofen application (500 nM to 100 μM) produced an 18 – 100% reduction in the amplitude of $[Ca^{2+}]_i$ transients evoked by local KCl applications (25 mM) in 51 cells (Figure 4A). The concentration-response curve for baclofen showed an $EC_{50} = 1.8$ μM (Figure 5, *cf.* Table 2).

To ascertain a $GABA_B$-receptor involvement in the baclofen-mediated response, we applied a $GABA_B$-receptor antagonist before, or during the application of baclofen to

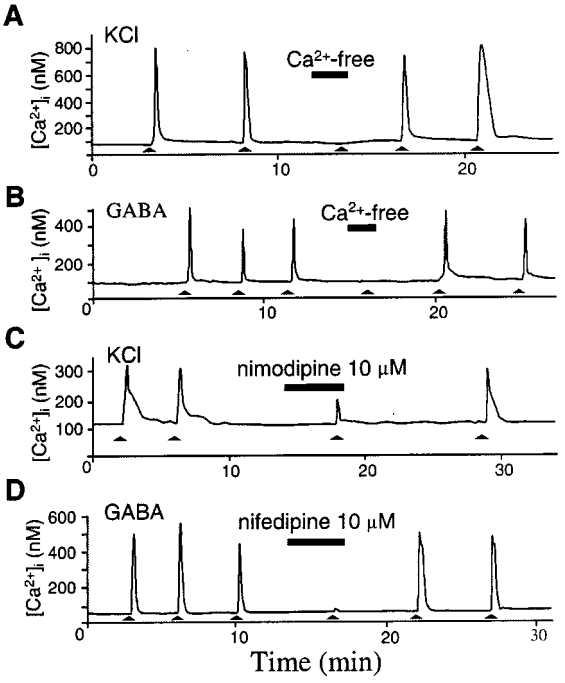

Figure 3 Involvement of voltage-dependent Ca^{2+}-channels evoked by GABA (100 μM) and KCl (25 mM) in 4 cells (A–D). (A) Local application of KCl (indicated by arrowheads) increased $[Ca^{2+}]_i$ but did not raise $[Ca^{2+}]_i$ during perfusion with Ca^{2+}-free media (bar). (B) GABA application (indicated by arrowheads) also did not evoke a response during perfusion with Ca^{2+}-free media, implying an extracellular Ca^{2+}-requirement. (C) Application (bar, 5 min) of nimodipine (10 μM) reversibly inhibited the KCl-evoked increase in $[Ca^{2+}]_i$. (D) Application (bar, 5 min) of nifedipine (10 μM) reversibly attenuated the GABA-evoked increase in $[Ca^{2+}]_i$.

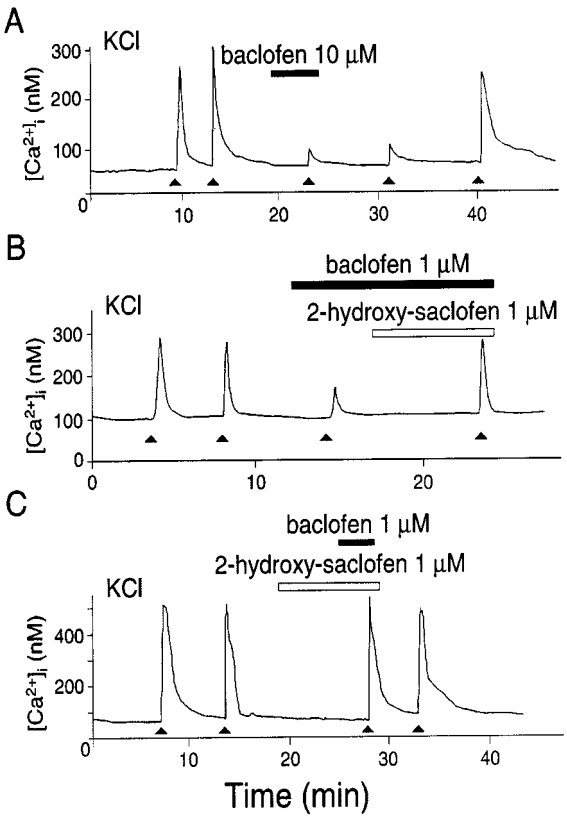

Figure 4 Effects of $GABA_B$-receptor activation in three cells (A–C), demonstrated by the bath applications (bars) of baclofen and antagonist, 2-hydroxy-saclofen, on the increases in $[Ca^{2+}]_i$ induced by local application of KCl (25 mM) and indicated by arrowheads. (A) Baclofen application (10 μM, 4 min) inhibited the increases in $[Ca^{2+}]_i$ induced KCl. (B) Baclofen application (1 μM) produced >50% inhibition of the KCl-induced peak elevation of $[Ca^{2+}]_i$ but was ineffective in producing inhibition during co-application with 2-hydroxy-saclofen (1 μM, 7.5 min). (C) After two control responses to a high extracellular $[K^+]$ and a pretreatment with 2-hydroxy-saclofen (1 μM) for ~6 min, a subsequent application of baclofen (1 μM) did not inhibit the KCl-evoked increase in $[Ca^{2+}]_i$. On termination of the baclofen and 2-hydroxy-saclofen co-application, a KCl-induced elevation of $[Ca^{2+}]_i$ was apparent several min later.

the cells. Bath applications of 2-hydroxy-saclofen for 4–8 min had little effect on the resting $[Ca^{2+}]_i$ but antagonized the effects of baclofen (500 nM–10 μM) on the KCl-evoked increase in $[Ca^{2+}]_i$ ($n=18$; Figure 4B,C). Prior to administration of 2-hydroxy-saclofen, baclofen decreased the KCl-induced elevations in $[Ca^{2+}]_i$ by 25–85% ($n=11$). After a continuous application of baclofen reduced the KCl-responses, a subsequent co-application of baclofen and 2-hydroxy-saclofen (1–20 μM) did not result in a depression of the KCl response (Figure 4B). When the cells were pretreated with 2-hydroxy-saclofen for 5 min, co-applied baclofen did not greatly affect the amplitude or duration of the $[Ca^{2+}]_i$ responses evoked by applications of KCl ($n=33$; Figure 4C). From the above results it was evident that MD3 cells possessed $GABA_B$-receptors in addition to $GABA_A$-receptors.

Discussion

These studies have delineated the effects of GABA on the $[Ca^{2+}]_i$ of a cell line derived from murine DRG neurones and N18TG2 neuroblastoma cells. The MD3 cells expressed similarities to the parent neurones, such as MAP-2 and neurofilament immunoreactivity (Naves *et al*., 1996). Most cells exhibited a transient increase in $[Ca^{2+}]_i$ upon application of α,β-CH_2-ATP. Approximately half of the cells exhibited capsaicin sensitivity. The responses to α,β-CH_2-ATP required a normal extracellular $[Ca^{2+}]$ whereas capsazepine application prevented the cells from responding to capsaicin. These effects are consistent with the presence of vanilloid and purinergic receptors on DRG neurones (Rae *et al*., 1998; Gschossmann *et al*., 2000). It is likely that the sensitivities to capsaicin and α,β-CH_2-ATP identify approximately half of the MD3 cells as a heat-reactive nociceptive class of cells (Petruska *et al*., 2000). This classification still would include several functional cell types whereas the remainder of MD3 cells likely represents a heterogeneous population with functionally distinct features, including different sensory modalities and sensitivities to GABA and many other modulators.

GABA-receptor activation

Similar to the parent neurones, MD3 cells showed responses attributable to activations of $GABA_A$ and $GABA_B$ receptors. Previous investigators have reported that many substances, thought to modulate neuronal function, either elevated or

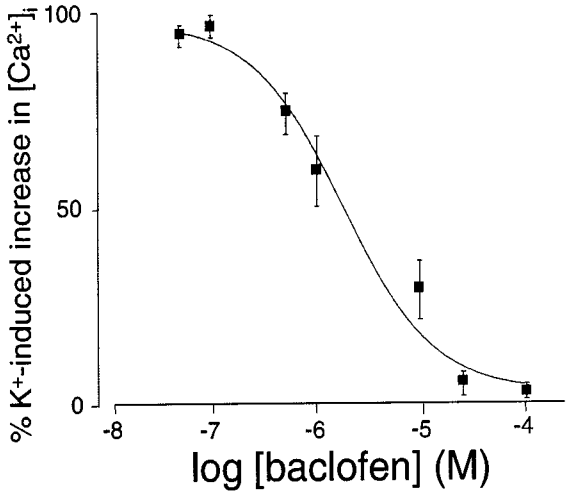

Figure 5 Concentration-response relationship for baclofen-mediated inhibition of the increase in $[Ca^{2+}]_i$ evoked by local application of KCl. After the control responses, bath application of baclofen (50 nM – 100 μM) reduced the KCl-evoked increases in $[Ca^{2+}]_i$. This inhibition was calculated as a percentage of two averaged controls. The ED_{50} for baclofen was 1.8 μM. The relationship between the per cent mean effect and the logarithm of the baclofen concentration was fitted with a sigmoidal function using GraphPad software. Data represent per cent KCl-induced increase in $[Ca^{2+}]_i$ (mean ± s.e mean, Table 2).

Table 2 Effects of baclofen on KCL-induced increase in $[Ca^{2+}]_i$

[Baclofen] (μM)	$[Ca^{2+}]_i$ (% of control)	P *value*	n
0.5	94.0 ± 2.8	n.s.	19
0.1	96.7 ± 2.8	n.s.	18
0.5	74.3 ± 5.2	< 0.01	15
1	59.6 ± 8.8	< 0.01	14
10	28.8 ± 7.6	< 0.0001	13
25	4.6 ± 3.0	< 0.0001	6
100	0.8 ± 0.5	< 0.0001	4

Inhibition of KCl-evoked peak elevations in $[Ca^{2+}]$i was measured at 4 min of baclofen application (bath). Inhibition of the KCl-evoked response was calculated as a fraction of control values that were an average of two or more peak elevations in $[Ca^{2+}]$i due to local KCl (25 mM) applications. The values are expressed as per cent of control amplitude (mean ± s.e.mean; n.s. = non-significant). The *P* values indicate statistical differences from control values (Student's *t*-test). '*n*' refers to the number of experiments studied at a given concentration.

produced no changes in the $[Ca^{2+}]_i$ of DRG neurones (Bowie *et al*., 1994). To the best of our knowledge, there are no previous reports of GABA's effects on $[Ca^{2+}]_i$ in DRG neurones.

MD3 cells responded to GABA agonists with rapid elevations in $[Ca^{2+}]_i$ that typically peaked 300 – 400% greater than the resting $[Ca^{2+}]_i$. The responses were approximately twice as large as in cultured embryonic rat dorsal horn neurones (Reichling *et al*., 1994) or adrenal chromaffin cells (Kitayama *et al*., 1990) but > 10 fold smaller than observed in a pituitary gonadotrope cell line (Williams *et al*., 2000). Application of ZAPA, a potent $GABA_A$-receptor agonist, produced a large, transient increase in $[Ca^{2+}]_i$ in 24 responding MD3 cells. As demonstrated in oligodendrocyte precursor cells, rat brain membranes and guinea-pig ileum (Allan *et al*., 1997; Williamson *et al*., 1998), the effects of ZAPA implied that $GABA_A$-receptors exist on MD3 cells.

Effects of $GABA_A$-receptor antagonists

We confirmed a $GABA_A$-receptor involvement in MD3 cells by observing that bicuculline application reversibly eliminated the $[Ca^{2+}]_i$ response to GABA. The antagonism by bicuculline, with an $IC_{50} = 9.3$ μM, is consistent with its interactions with GABA at $GABA_A$-receptors (Macdonald & Olsen, 1994).

On the other hand, picrotoxinin application did not block the GABA responses in MD3 cells. The GABA-evoked inward currents of adult human DRG neurones in culture are insensitive to picrotoxinin as well as bicuculline (Valeyev *et al*., 1996) whereas these agents antagonize the GABA-currents in human embryonic DRG and rat DRG neurones, cultured under identical conditions (Valeyev *et al*., 1995; 1999). Both antagonists do not greatly affect the GABA-responses in cultured hippocampal neurones (Segal, 1993). Neuronal responses to GABA in slices of the developing rat hippocampus and in isolated frog midbrain are sensitive to blockade with picrotoxinin, but not bicuculline (Nistri & Sivilotti, 1985; Strata & Cherubini, 1994). In summary, several investigators have reported a refractoriness or relative insensitivity of GABA-responses to $GABA_A$-antagonists in DRG and CNS neurones. As inferred from these reports, the picrotoxinin-insensitivity in MD3 cells may result from developmental or species differences in the functional organization of the $GABA_A$-receptor; these effects could arise from variations in the levels of subunit mRNA encoding, or from the composition or stoichiometry of $GABA_A$-receptor subunits (Gambarana *et al*., 1990; Strata & Cherubini, 1994; Shen *et al*., 1999).

Diazepam insensitivity

Analogous variations in the benzodiazepine receptor (Macdonald & Olsen, 1994; Williamson *et al*., 1998) may account for the failure of diazepam to potentiate GABA actions in MD3 cells and dorsal roots (Wesselman *et al*., 1991). Whereas diazepam potentiates GABA-induced responses in adult human DRG neurones (Valeyev *et al*., 1996), MD3 cells may lack an appropriate subunit combination (e.g., α4 or α6 and ρ subunits) for $GABA_A$ receptor-interactions with diazepam (Cherubini & Conti, 2001).

Mechanism of GABA-action

The rise in $[Ca^{2+}]_i$ initiated by GABA at $GABA_A$-receptors in MD3 cells involved a Ca^{2+} influx. We infer this from observations that perfusion with nominally Ca^{2+}-free media eliminated the changes in $[Ca^{2+}]_i$ induced by application of a $GABA_A$-agonist. In several CNS preparations, $GABA_A$-receptor activation also produces a Ca^{2+} influx (Segal, 1993; Reichling *et al*., 1994; Owens *et al*., 1996; Canepari *et al*., 2000).

In view of possible internal Ca^{2+} modulation or Ca^{2+} dependence of the responses, care was taken in our

experiments to limit the exposure of the cells to Ca^{2+}-free media so as not to deplete intracellular Ca^{2+} stores. In retinal ganglion cells, intracellular Ca^{2+} may modulate $GABA_A$-mediated currents through one or more protein phosphatases (Akopian *et al*., 1998). Application of thapsigargin, an inhibitor of intracellular Ca^{2+} pumps, or caffeine, which releases Ca^{2+} from internal stores, did not greatly change the resting $[Ca^{2+}]_i$ in MD3 cells. Such observations may not be unusual for some cells (Guerini & Carafoli, 1999). We did not observe significant effects of thapsigargin or caffeine on the increase in $[Ca^{2+}]_i$ evoked by GABA. Hence, the rise in $[Ca^{2+}]_i$ initiated by GABA at $GABA_A$-receptors did not seem to involve Ca^{2+} release from internal stores in MD3 cells.

The return of elevated $[Ca^{2+}]_i$ to baseline values may have resulted from the activities of a plasma membrane Ca^{2+}-ATPase mechanism (Usachev *et al*., 2001). We observed a plateau in the return of $[Ca^{2+}]_i$ from the peak to the resting value in some cells after GABA or KCl application (*cf*. Figure 3C). This slower decline in the recovery phase of the $[Ca^{2+}]_i$ transient may result from mitochondrion-mediated $[Ca^{2+}]_i$ buffering, as in cultured DRG neurones (Baron & Thayer, 1997).

In DRG and CNS neurones, $GABA_A$-receptor activation increases membrane conductance for Cl^- (Macdonald & Olsen, 1994; Alvarez-Leefmans *et al*., 1998). This results in a depolarization of DRG somata and terminations as well as the dendrites and nerve terminals of some CNS neurones because of an outwardly directed Cl^- gradient, maintained by a Na^+-dependent Cl^- transporter (Cherubini *et al*., 1991; Reichling *et al*., 1994; Alvarez-Leefmans *et al*., 1998). DRG neurones from mice with a disrupted gene that normally encodes the Na-K-2Cl co-transporter do not exhibit depolarizing responses to GABA (Sung *et al*., 2000). In contrast, DRG neurones from wild type mice of the same age have depolarizing responses to GABA and a significantly more depolarized reversal potential for GABA action (E_{GABA}) than in the Na-K-2Cl co-transporter (NKCC1) null mice. This implies that the NKCC1 is responsible for adjusting the internal $[Cl^-]$ in DRG neurones (Sung *et al*., 2000) and neuroblastoma cells (Sun & Murali, 1998). It seems likely that modulatory factors in the developing animal down-regulate Na-K-2Cl activity resulting in a progressively more negative E_{GABA} as the animal matures. In cortical and dorsal horn neurones, $GABA_A$-receptor activation results in a depolarization during mammalian embryogenesis (Reichling *et al*., 1994) or early postnatal life (Owens *et al*., 1996). From our results, we suggest that the N-K-2Cl co-transporter activity is high in MD3 cells, leading to persistently elevated internal $[Cl^-]$.

High-voltage activated Ca^{2+}-channels

We investigated the hypothesis that $GABA_A$-receptor activation and a subsequent depolarization resulted in increased voltage-dependent Ca^{2+} channel activity which lead to an increased $[Ca^{2+}]_i$. Applications of 25 mM KCl increased $[Ca^{2+}]_i$, similar to the effects of GABA agonists. Using the Goldman-Hodgkin-Katz equation, we calculated that this external $[K^+]$ would depolarize neurones by >40 mV, under the experimental conditions. Assuming that the resting membrane potentials were in a range of -50 to -60 mV, we would expect a depolarization of this magnitude to open high-voltage-activated Ca^{2+} channels. The application of nifedipine or nimodipine attenuated or completely blocked the KCl-induced elevations of $[Ca^{2+}]_i$. Both dihydropyridines also attenuated the GABA-induced elevations of $[Ca^{2+}]_i$ in MD3 cells, as in cultured dorsal horn neurones (Reichling *et al*., 1994) and gonadotrope cells (Williams *et al*., 2000). Reichling *et al*. (1994) did not find evidence for the GABA-activation of cation-permeable channels, in addition to Cl^--permeable channels. It seemed likely that $GABA_A$-receptor activation depolarized MD3 cells by initiating a Cl^- efflux; this depolarization evoked a Ca^{2+} influx through high voltage-activated Ca^{2+} channels, thereby elevating $[Ca^{2+}]_i$. $GABA_A$-receptor activation may then have secondary effects on second messengers and metabolic processes.

$GABA_B$-receptor activation

We also found evidence for functional $GABA_B$-receptors in MD3 cells. Without affecting the resting $[Ca^{2+}]_i$, baclofen inhibited the KCl-induced elevations of $[Ca^{2+}]_i$. Application of 2-hydroxy-saclofen itself did not greatly affect the KCl-induced responses but completely blocked the baclofen-inhibition of the responses to KCl. The observed effects of baclofen at $GABA_B$-receptors may have resulted from a decrease in voltage-activated Ca^{2+} currents (Robertson & Taylor, 1986; Bowery, 1993).

Given the similarities of MD3 cells to DRG neurones, the effects of baclofen and the antagonism by 2-hydroxy-saclofen imply that $GABA_B$-receptor activation can influence voltage-dependent activities in sensory ganglia *in vivo*. The effects resulting from $GABA_B$-receptor activation in DRG neurones *in vivo* would depend on somatic invasion of action potentials into the cell bodies and their involvement in the activities of peripheral and central terminations (*cf*. Introduction).

Model of presynaptic inhibition

Many studies have employed DRG somata for modelling GABA-receptor interactions on primary afferent terminals in the CNS (Alvarez-Leefmans *et al*., 1998). This has resulted in a generally accepted shunt-model of presynaptic inhibition and an explanation of GABA modulation of nociceptor activity at peripheral sensory nerve endings (Carlton *et al*., 1999). In the model, the increased Cl^- conductance due to $GABA_A$-receptor activation decreases action potential invasion of the nerve terminals and reduces Ca^{2+} influx, curtailing transmitter release (Rudomin & Schmidt, 1999). The primary afferent depolarization would contribute to the reduction in transmitter release by inactivating Ca^{2+} currents and, would increase $[Ca^{2+}]_i$ in nerve terminal Ca^{2+} domains, distinct from the local domains associated with transmitter release (*cf*. Chad & Eckert, 1984).

In conclusion, immortalized MD3 cells possess properties of the parent DRG neurones, including functional $GABA_A$- and $GABA_B$-receptors. Similar to K^+ depolarization, $GABA_A$-receptor activation transiently elevated $[Ca^{2+}]_i$, probably by initiating Ca^{2+} influx through high voltage-activated channels. $GABA_B$-receptor activation reduced or abolished depolarization-initiated elevations in $[Ca^{2+}]_i$, likely by interfering with Ca^{2+}-channel function. The MD3 cells

provide a simple, reproducible model system for studying drug effects on the properties of DRG neurones and may have value for drug development.

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