



GABA_B mediated regulation of sympathetic preganglionic neurons: pre- and postsynaptic sites of action

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Modulatory influences on sympathetic nervous system activity are diverse and far reaching, acting at select points in the complex pathways controlling sympathetic outflow to enable subtle changes or more global effects. Changes in the degree of sympathetic neuromodulation can have serious consequences on homeostatic variables such as heart rate, blood pressure and gut motility. At the level of the spinal cord, the sympathetic preganglionic neurons (SPNs) can be modulated by activation of presynaptic GABA_B heteroreceptors on glutamatergic terminals and by postsynaptic GABA_B receptors. Here we show that a low concentration of the GABA_B agonist baclofen (1 μM) attenuated GABAergic inhibitory postsynaptic potentials in SPNs elicited from stimulation of either the central autonomic area or descending fibers in the lateral funiculus. This low baclofen concentration also elicited three categories of postsynaptic response: a large hyperpolarization with a decrease in input resistance, a moderate hyperpolarization with no change in input resistance and no response. Using cesium-loaded, tetraethylammonium chloride containing electrodes (to block potassium conductance), baclofen elicited moderate hyperpolarizations with no change in input resistance in 50% of SPNs; the remainder were unaffected. These modest hyperpolarizations were reduced in Ca²⁺ free solution or cadmium. Hyperpolarizing responses were also observed in interneurons in the vicinity of SPNs. These studies provide the first evidence for GABA_B autoreceptors involved in inhibitory GABAergic transmission onto SPNs and for postsynaptic GABA_B receptors on interneurons. The data also indicate that there is heterogeneity in the postsynaptic responses of SPNs.

Keywords: sympathetic, spinal cord, interneuron, GABA_B, baclofen, electrophysiology

INTRODUCTION

Sympathetic outflow to the many organs controlled by the autonomic nervous system is regulated at a number of levels within the central nervous system. The final point at which sympathetic activity can be influenced by the CNS is at the spinal cord level where the sympathetic preganglionic neurons (SPNs) reside. Continuing research into the spinal cord circuitry that regulates sympathetic output has revealed a high degree of complexity of control of these SPNs which enables fine tuning of the sympathetic activity influencing end organs. For example, studies from our laboratory have revealed that at least two GABAergic inhibitory influences on SPNs exist; one a descending monosynaptic inhibitory influence originating in the brainstem (Deuchars et al., 1997; Deuchars et al., 2001a) and another more local input from GABAergic interneurons located in the central autonomic area of the spinal cord (Deuchars et al., 2005). Thus one avenue for selective regulation of SPN activity would be at a presynaptic level since receptors located on specific presynaptic terminals would influence synaptic transmission exclusively from that input. Such input-specific modulation is observed in SPNs where A1 adenosine receptors are located on excitatory terminals and their activation decreases excitatory transmission (Deuchars et al., 2001a) whilst A2A adenosine receptors are confined solely to inhibitory GABAergic terminals and increase GABAergic transmission (Brooke et al., 2004).

GABA_B receptors, like adenosine receptors, are also important modulators of neuronal excitability (Kerr and Ong, 1995). Their widespread actions and involvement in mechanisms underlying pathologies such as epilepsy and chronic pain (Bowerly, 2006) have led to an increased interest in regulating these receptors as potential therapeutic targets. GABA_B receptors are present postsynaptically on SPNs and high concentrations of the GABA_B agonist, baclofen (10–100 μM) elicited hyperpolarizing responses associated with a decrease in input resistance that was attributed to opening of potassium channels (Whymant et al., 2004). However, a proportion of these responses may be due to activation of other second messenger mediated channels since in splanchnic SPNs, 5 μM baclofen also increased potassium channel opening but with an accompanying decrease in voltage gated calcium conductance, that together caused inconsistent effects on input resistance (Cheng et al., 2005). These discrepancies in findings regarding the nature of postsynaptic GABA_B mediated responses may be due to differences in concentration of baclofen used.

Presynaptic GABA_B heteroreceptors have been reported on excitatory terminals onto SPNs by Cheng's group and others (Cheng et al., 2005; Wu and Dun, 1992). Here applications of baclofen (1–30 μM) decreased excitatory amino acid mediated transmission from lateral funiculus (Lf) stimulation (which comprises descending inputs to SPNs) although Wu and Dun (1992) reported that baclofen was without effect on the postsynaptic membrane.

However, to date no evidence exists of GABA_B autoreceptors on descending or local inhibitory inputs onto SPNs. Such autoreceptors are common in other regions of the CNS and may display different pharmacological profiles to heteroreceptors (Bonanno and Raiteri, 1992). In fact, differences in the sensitivity of pre versus postsynaptic receptors to baclofen are also common findings – in neurons of the nucleus tractus solitarius, among others, the sensitivity of presynaptic receptors was at least 20-fold higher than that of the postsynaptic receptors (Brooks and Glaum, 1995).

The lack of data regarding a role for GABA_B autoreceptors in spinal cord circuitry underlying sympathetic control led us to examine how GABA_B receptors may affect synaptic inhibitory transmission from both descending and local inputs onto SPNs. Furthermore the intriguing possibility of GABA_B receptors with specific sensitivities to baclofen prompted us to determine how very low concentrations of baclofen affects both pre- and postsynaptic GABA_B receptors on SPNs in the thoracic spinal cord slice preparation.

MATERIALS AND METHODS

PATCH CLAMP RECORDINGS

All experiments were performed under UK Home Office License and in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986.

Wistar rats of either sex aged 10–15 days were anesthetized with urethane (2 g/kg, i.p.) and transcardially perfused with ice-cold sucrose aCSF, composition (in mM): sucrose (217), NaHCO₃ (26), KCl (3), MgSO₄ (2), NaH₂PO₄ (2.5), CaCl₂ (1), and glucose (10). After decapitation, the upper and middle thoracic spinal cord was isolated and the dura and pia mater were carefully removed. Coronal slices of 300 μm were cut using a Vibroslice (Campden Instruments) and kept in a holding chamber containing aCSF, composition (in mM): NaCl (124), NaHCO₃ (26), KCl (3), MgSO₄ (2), NaH₂PO₄ (2.5), CaCl₂ (2), and glucose (10), bubbled in 95% O₂/5%CO₂.

Slices were transferred to a submerged recording chamber and continually perfused with aCSF also containing the non-selective glutamate receptor antagonist kynurenic acid (2 mM) at a rate of 2–5 ml/min at room temperature. For experiments carried out in calcium-free aCSF, CaCl₂ was replaced with 2 mM MgCl₂. Whole-cell patch clamp recordings were made in current-clamp mode using an Axopatch 1D (Axon Instruments, Foster City, CA, USA). Patch electrodes (tip diameter, 3 μm; resistance 3–6 MΩ) were filled with either K-Gluconate based intracellular solution, composition (in mM): K-Gluconate (110), EGTA (11), MgCl₂ (2), CaCl₂ (0.1), HEPES (10), Na₂ATP (2), and NaGTP (0.3); or Cs-based intracellular solution, composition (in mM): Cs₂SO₄ (110), CaCl₂ (0.5), MgCl₂ (2), EGTA (5), HEPES (5), ATP-Na₂ (5), TEA (5). Liquid junction potentials (14.8 mV for K-Gluconate intracellular; 10.9 mV for Cs₂SO₄ intracellular) were not corrected for in these experiments.

Neurons (SPNs and interneurons) within the IML were identified both electrophysiologically and anatomically as described previously (Deuchars et al., 2001b). When using Cs-based intracellular solution, the electrophysiological properties of the two populations were less prominent but their distinctive morphology still enabled identification *post hoc*. Depolarizing current pulses of +10 to +200 pA were applied to examine firing properties of the neurons.

Responses to bath applied drugs were determined. Neurons were held at –60 mV (not corrected for liquid junction potentials) to test the effects of the GABA_B agonist baclofen and hyperpolarizing current pulses of –10 to –50 pA (1 s duration) injected to monitor changes in input resistance, represented by a change in the size of the voltage response to the injected current. Baclofen was repeatedly applied and during a response, the membrane potential was returned to –60 mV to check that the change in input resistance was due to an effect of the drug and not the voltage induced changes. To confirm that changes in membrane potential and input resistance were due to a specific action of the agonist at the GABA_B receptor, the selective antagonist CGP55845 was applied for ≥5 to 10 min prior to application of the agonist.

The central autonomic area and the Lf were stimulated using bipolar stimulating electrodes and isolated stimulators (DS2A; Digitimer, Hertfordshire, UK). IPSPs were elicited at more depolarized potentials (–10 to +10 mV) to increase the chloride driving force. Paired pulse stimuli were used to investigate presynaptic actions of pharmacological tools.

Drugs, a GABA_B agonist baclofen and an antagonist CGP55845 (both from Tocris Cookson, Bristol, UK), were applied in the superfusing solution and the concentration given is the final concentration in the bath. Both drugs were first dissolved in DMSO and the final concentration of DMSO in the bath was less than 1:1000. Bicuculline hydrochloride (Tocris Cookson) and cadmium chloride (Sigma, UK) were dissolved in water.

DATA ANALYSIS

Data capture was carried out using either Signal (version 1) and Spike 2 (version 2; Cambridge Electronic Design) or pClamp (version 9, Axon Instruments). The input resistance was calculated for each neuron by measurement of the voltage responses to injected current pulses (average of 3 sweeps). IPSP amplitudes were calculated by measuring their peak amplitude from the holding potential and were averaged for ≥10 consecutive sweeps and are given as the mean ± SEM.

Changes in IPSP amplitude as a consequence of drug application were expressed as a mean percentage of the control. Assessment of changes in the paired pulse ratio (PPR) was also carried out. PPR was derived from the ratio of the amplitude of the second IPSP to the first IPSP. The PPR during drug application was then compared with the control to note any significant change. The effects of the drugs were tested statistically using the paired Student's *t*-test, and differences were considered significant when *p* < 0.05.

RNA EXTRACTION AND PCR

Total RNA was isolated from micropunches of IML and spinal cord slices of rats (10–15 days), anesthetized and prepared as described above. The RNA was reverse transcribed as previously described (Spary et al., 2008). PCR was performed with 0.4 μM primers (GABA_{B1a} forward 5'-TAGCGATCGAGAAGGGGAGA-3'; GABA_{B1a} reverse 5'-CTTCACCTGGTCGCGAGTCA-3'; GABA_{B1b} forward 5'-GCGAACGTCTCTGGCCCTA-3'; GABA_{B1b} reverse 5'-GCGGGAGATGAGGGGAGTGA-3'; GABA_{B2} forward 5'-AGCAGATCCGCAACGAGTCA-3'; GABA_{B2} reverse 5'-GTGCTTCAGGAGCTTCAGGA-3') (Spary et al., 2008).

Negative controls included dH₂O and RNA, which had not undergone reverse transcription. The veracity of the PCR products was confirmed by DNA sequencing on an ABI 3100 genetic analyzer using BigDye terminator cycle sequencing version 3.1.

RESULTS

POSTSYNAPTIC EFFECTS OF LOW CONCENTRATIONS OF BACLOFEN RECORDED IN K-GLUCONATE BASED INTRACELLULAR SOLUTION

Firstly we determined how a low concentration of the GABA_B receptor agonist baclofen affected postsynaptic responses of SPNs, using a concentration (1 μ M) that has previously been shown in the nucleus of the tractus solitarius to selectively target pre- rather than postsynaptic receptors (Brooks et al., 1992). Using K-Gluconate based intracellular solution, this low concentration of baclofen (1 μ M) elicited three types of responses in SPNs tested ($n = 32$), characterized by the degree of change in membrane potential and input resistance. Thus the SPNs were grouped according to their responses into (i) a large-response group (37.5%) where both parameters were significantly altered by drug application (Figure 1A); (ii) a modest response group (34.4%) with small hyperpolarizations observed but no significant alteration in input resistance (Figure 1B); and (iii) a non-responding group (28.1%) where neither membrane potential nor input resistance of neurons were changed after bath application of baclofen (Figure 1C).

In the large-response group, the average change in cell membrane potential was -7.24 ± 0.9 mV associated with a decrease in input resistance to $87.6 \pm 3\%$ of control (612.7 ± 83 to 535.2 ± 72 M Ω , $p < 0.005$, $n = 12$). Responses were also maintained in the presence of 1 μ M external TTX ($n = 3$) and antagonized by the GABA_B receptor antagonist CGP55845 (mean hyperpolarization in baclofen alone was -8.9 ± 1.5 mV, mean hyperpolarization in baclofen + CGP55845 was -0.2 ± 0.2 mV; $p < 0.005$, $n = 5$; Figure 3A and see also Wang et al., 2008). An early study in SPNs also reported large hyperpolarizations associated with decreases in input resistance (Whyment et al., 2004) when using much higher concentrations of baclofen (10–100 μ M) and suggested that this hyperpolarization was due to activation of GABA_B receptor coupled K conductances. The moderate response group only showed hyperpolarizations of -1.80 ± 2.4 mV ($n = 11$; Figure 1B) which were significantly smaller than the large response group ($p < 0.001$) and the input resistances of these cells were not significantly altered (597.6 ± 50 to 586.4 ± 49 M Ω). Non-responding cells were not affected by low concentrations of baclofen (average change in membrane potential was 0.24 ± 0.2 mV, $n = 9$; Figure 1C). A lower concentration (0.5 μ M) also showed a similar grouping of responses (data not shown) to these observed with 1 μ M baclofen but with smaller sample sizes.

Electrically coupled activities (observed as spikelets described by Logan et al., 1996; Nolan et al., 1999) were recorded from 10 of the 32 SPNs, where a strong degree of coupled activity was noted. These strongly coupled cells were equally distributed between the three subgroups (4, 4, 2, in three groups respectively). Coupled events in all four cells in the large-response group were completely abolished during drug application. In the moderate response group, baclofen completely blocked spikelets in 2/4 cells and left the other two partially reduced in frequency. In the non-responding group, without altering the membrane properties of the recorded neurons, baclofen blocked the spikelets of one strongly coupled cell and reduced the frequency of the other (Figure 1C).

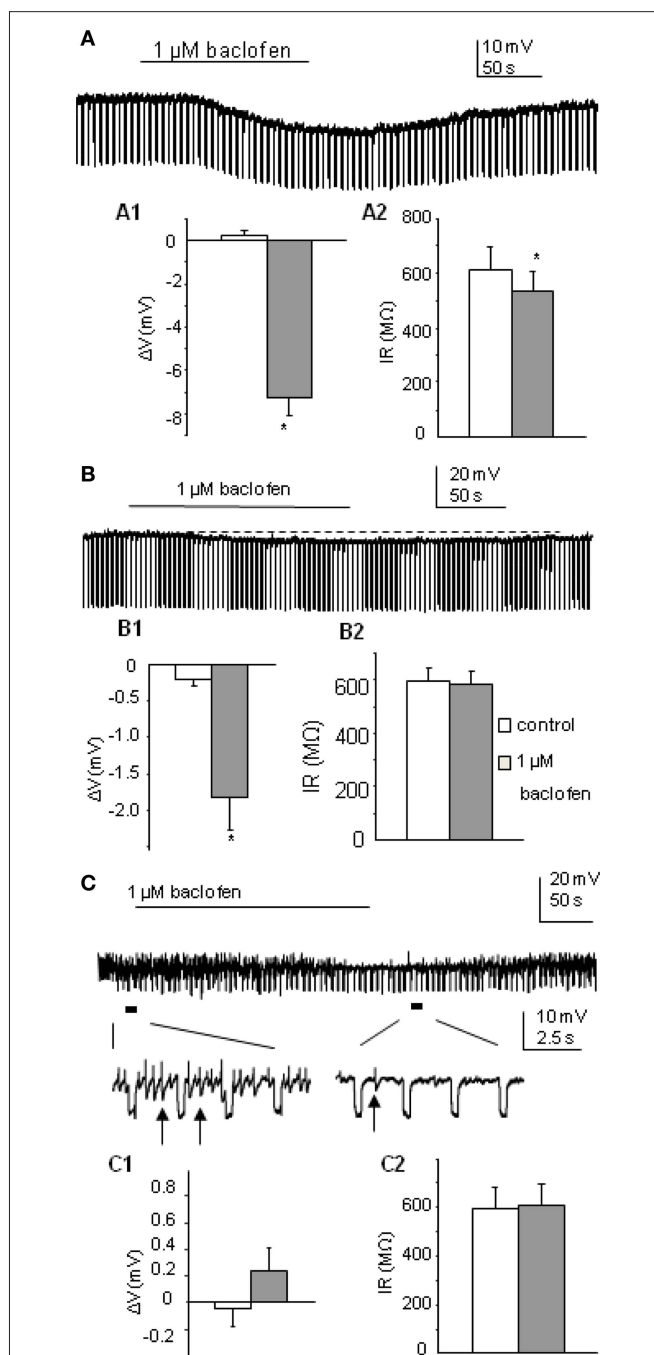


FIGURE 1 | Postsynaptic responses to 1 μ M baclofen differ in SPNs recorded in K-Gluconate based intracellular solution. (A) Bath application of baclofen induced hyperpolarization in this SPN, associated with a decrease in cell input resistance. **(A1,A2)** Pooled data ($n = 12$) showing membrane potential and input resistance changes before and during drug application. $*p < 0.05$, Student's t -test. **(B)** Bath application of baclofen hyperpolarized this SPN slightly without changing cell input resistance. **(B1,B2)** Pooled data ($n = 11$) showing membrane potential and input resistance changes before and during drug application. **(C)** Bath application of baclofen did not induce any membrane potential or input resistance changes in this SPN. Expanded traces showing coupled activities (arrows) were reduced but not abolished during drug application. **(C1,C2)** Pooled data ($n = 9$) showing membrane potential and input resistance changes before and during drug application.

POSTSYNAPTIC EFFECTS OF LOW CONCENTRATIONS OF BACLOFEN RECORDED IN Cs-BASED INTRACELLULAR SOLUTION

Cs loaded TEA containing intracellular solution was used to internally block K⁺ conductance. Therefore, any membrane potential changes due to opening of K⁺ channels associated with activation of GABA_B receptors should no longer be seen. TTX was also applied. Of the 18 neurons recorded, 50% were only slightly hyperpolarized to a mean value of -3.02 ± 0.3 mV (this is a significant change from control, $p < 0.005$; $n = 9$; **Figure 2A**) with no significant input resistance changes (346.0 ± 46 to 348.3 ± 46 M Ω), similar to that observed in the moderate response group with K-Gluconate based intracellular solution. Indeed there was no significant difference between these values and those moderate responses recorded in K-Gluconate, even though there were slight differences in liquid junction potentials between the two solutions. CGP55845 also antagonized the effects of baclofen with Cs-based intracellular solutions, similar to that observed with K-Gluconate containing electrodes (mean hyperpolarization to baclofen was -3.14 ± 0.5 mV, whilst in CGP + baclofen, the mean hyperpolarization was -0.02 ± 0.2 mV, $n = 3$; **Figure 3B**). The other SPNs did not respond to 1 μ M baclofen application (**Figure 2B**; $n = 9$).

Of the 18 cells recorded, 13 were strongly coupled with their neighboring cells. In the non-responding group, bath application of baclofen completely blocked all the spikelets in two cells and reduced the frequency of spikelets in one cell. Coupled activities from three other cells stayed unchanged. The remaining two cells of the group only displayed occasional spikelets therefore were not compared. All coupled activities from the seven coupled cells of the small response group were abolished by baclofen application.

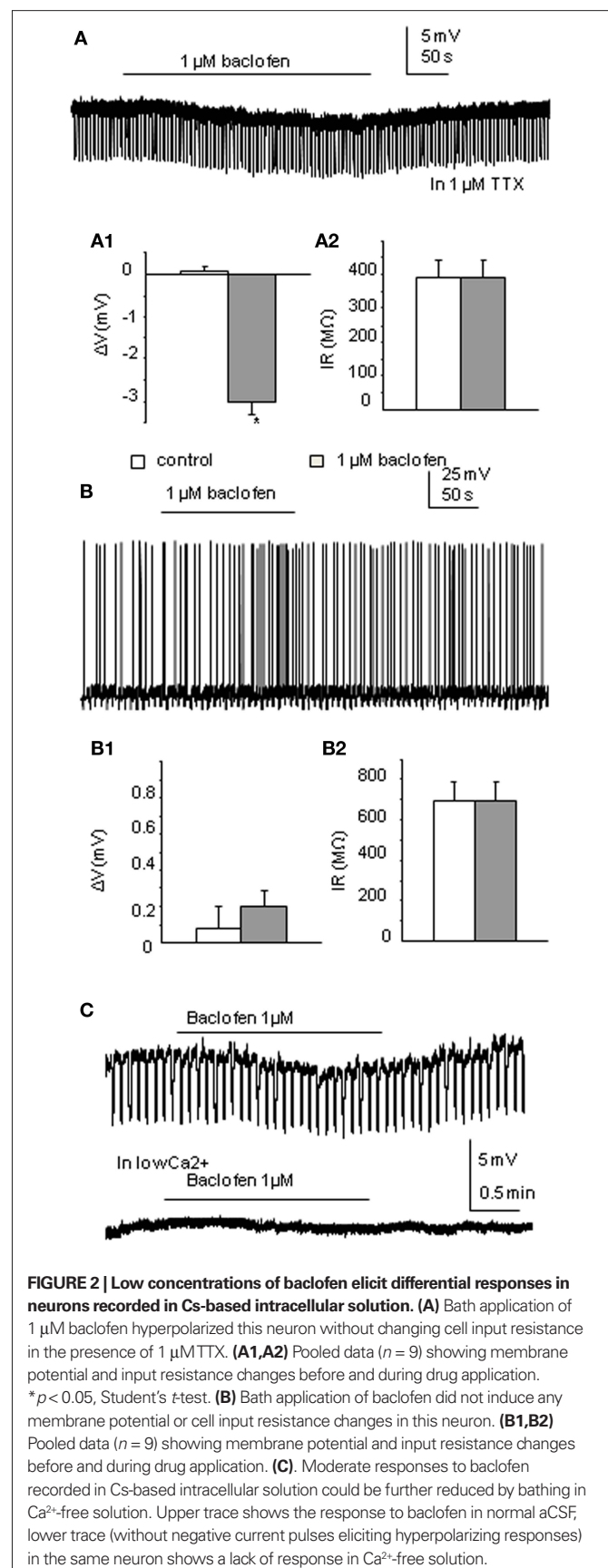
The effects of Ca²⁺-free solution or cadmium chloride (100 μ M) was tested on baclofen responses using Cs-based intracellular solution. Both Ca²⁺-free solutions and CdCl₂ significantly reduced the small hyperpolarizing effects of baclofen recorded in Cs intracellular solution from -2.3 ± 0.2 to -0.3 ± 0.1 mV ($p < 0.005$, $n = 8$; **Figure 2C**) with the extent of the blockade in the two situations not significantly different.

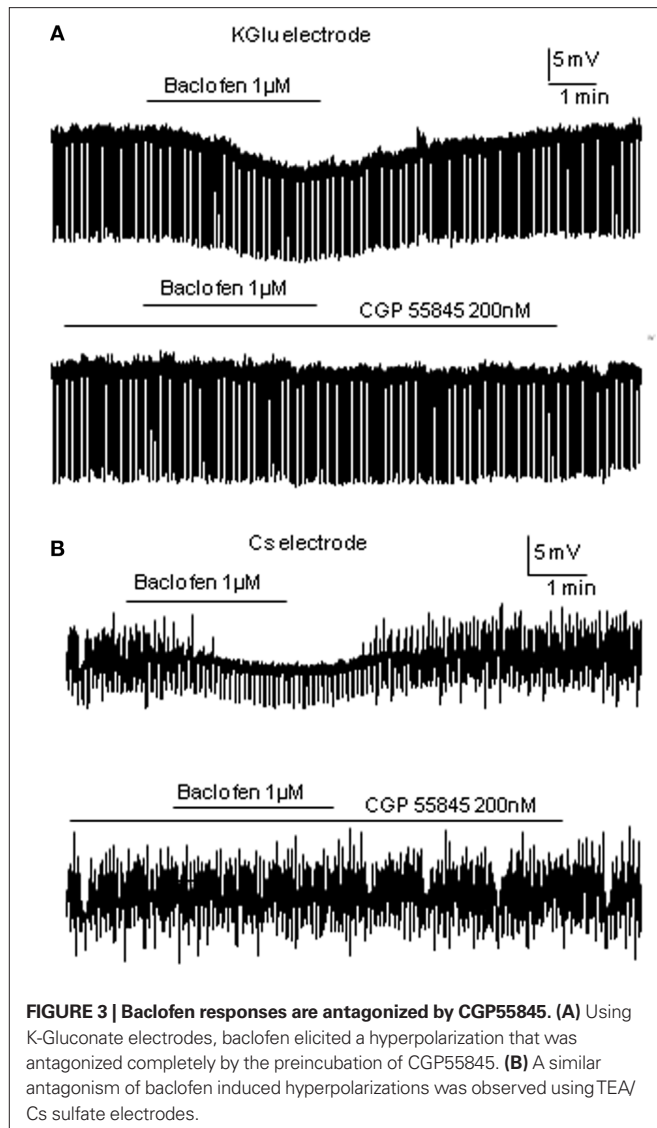
POSTSYNAPTIC EFFECTS OF LOW CONCENTRATIONS OF BACLOFEN ON INTERNEURONS

In all five interneurons recorded using K-Gluconate based intracellular solution, bath application of 1 μ M baclofen elicited an average membrane potential change to -7.2 ± 0.9 mV ($p < 0.005$), associated with a decrease in input resistance from 596 ± 102 to 463 ± 102 M Ω ($p < 0.05$; **Figure 4**). This indicates an effect of baclofen on GABA_B receptors in these cells. By comparing the input resistance changes of the two cell populations (large-response group of SPNs and interneurons), the decrease in input resistance induced by low concentration of baclofen in interneurons is significantly higher than that of SPNs in the IML ($p < 0.05$).

GABA_B SUBUNITS ARE EXPRESSED IN THE IML OF SPINAL CORD

To further verify the presence in the IML of the different subunits that may contribute to these effects, PCR was performed on punches of IML from spinal cord tissue of the same age, and prepared in the same way, as that used in the electrophysiological experiments.



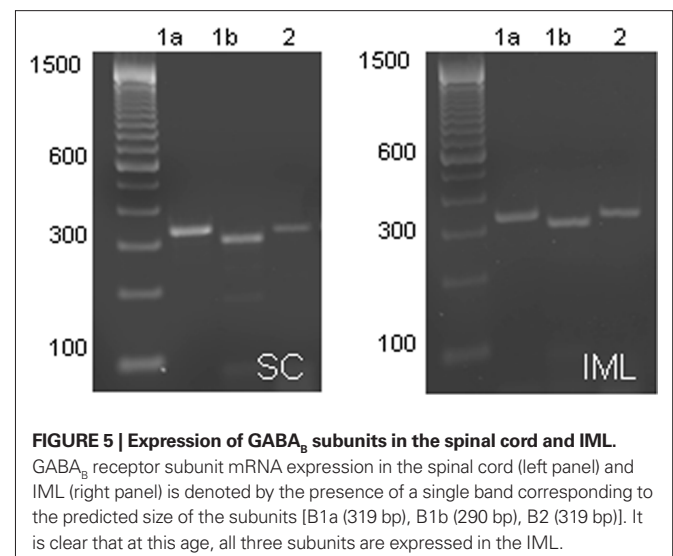
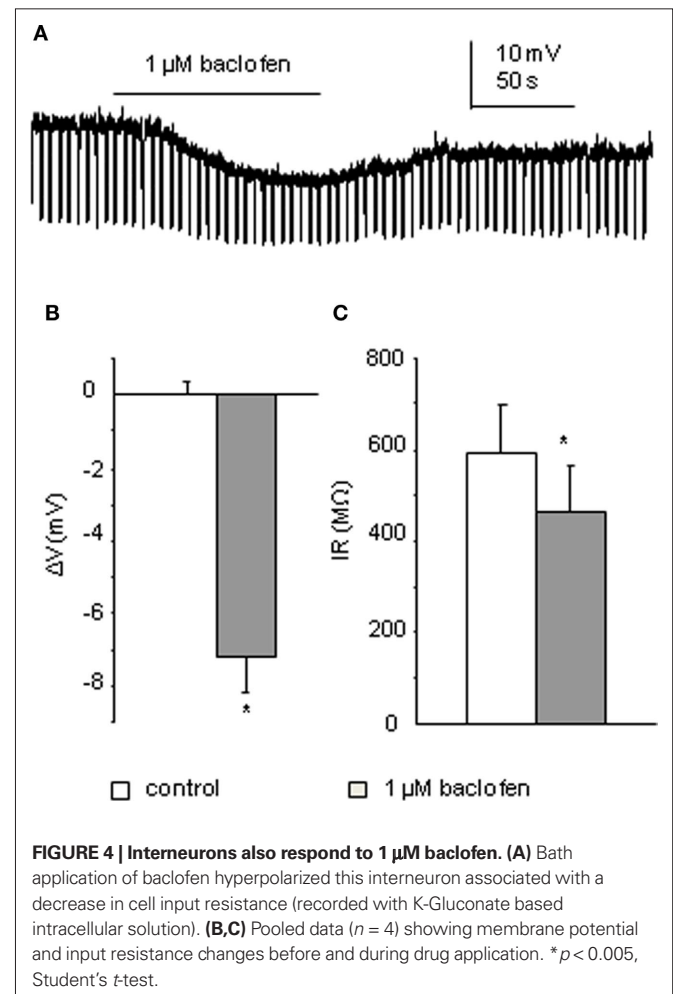


PCR analysis was performed on cDNA samples taken from the spinal cord and IML. Expression of all GABA_B subunits was observed in both the spinal cord and IML samples ($n = 4$; **Figure 5**), denoted by the presence of a single band corresponding to the predicted size of each subunit [B1a (319 bp), B1b (290 bp), B2 (319 bp)].

PRESYNAPTIC EFFECTS OF LOW CONCENTRATIONS OF BACLOFEN ON GABAERGIC SYNAPTIC TRANSMISSION

In addition to activating at postsynaptic sites, GABA_B receptors can also be present on presynaptic terminals as auto- or heteroreceptors. As presented above, blockade of potassium conductance with cesium eliminated the postsynaptic receptor mediated large response by 1 μ M baclofen. Under these conditions, regardless of the membrane potential responses, which comprise either no effect or small hyperpolarizations, the whole-cell input resistance remains unchanged. Therefore with Cs intracellular solution, any alterations observed in evoked GABAergic IPSPs would likely be due to a presynaptic modulatory effect. A paired pulse stimulation protocol was used in both Lf and CAA monosynaptic GABAergic pathways in order to monitor the changes in PPR.

Bath application of 1 μ M baclofen significantly reduced the peak amplitude of both CAA and Lf-IPSPs. The mean amplitudes of first IPSPs evoked via Lf stimulation were decreased to $75.4 \pm 3.9\%$ and



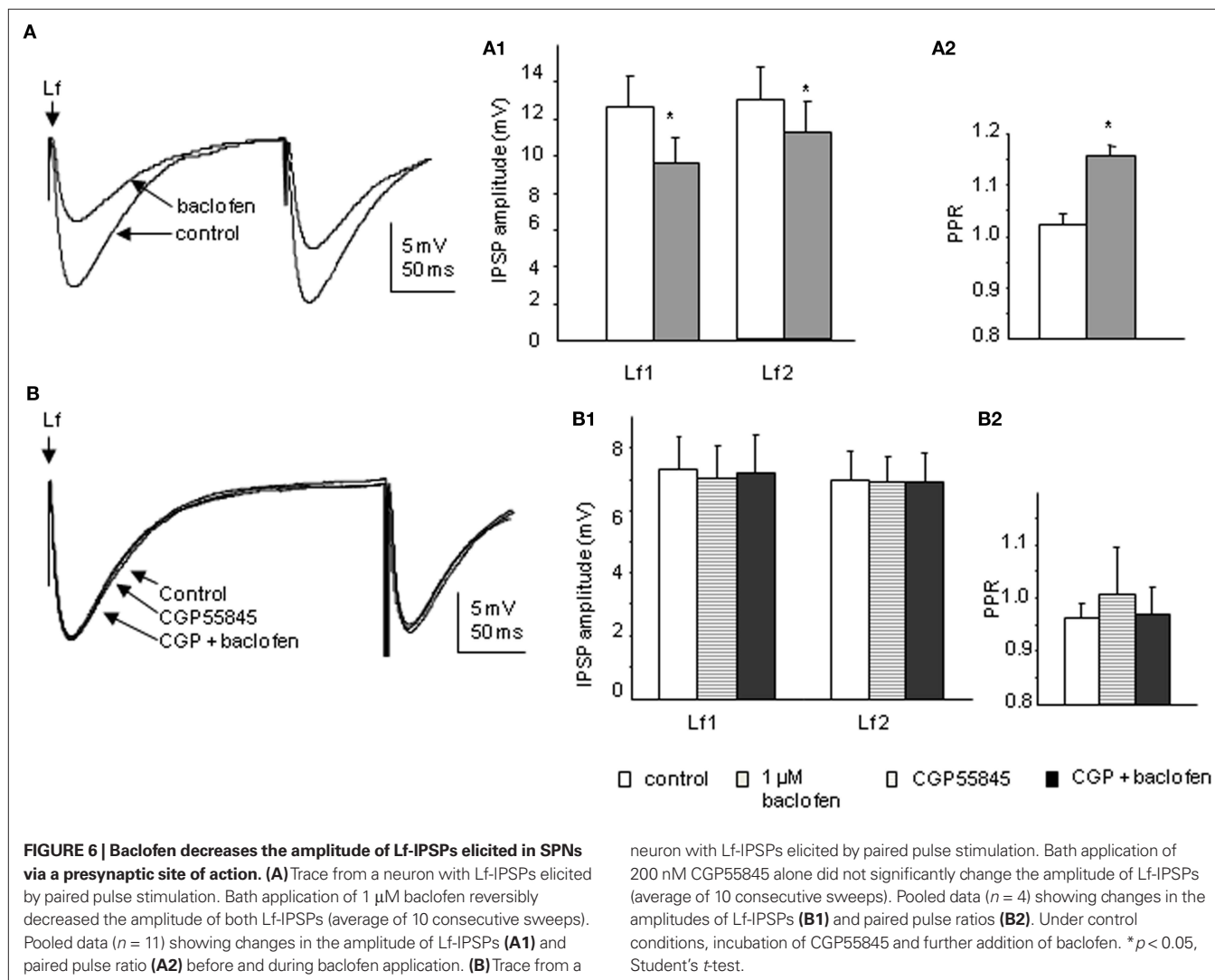
the second IPSPs to $83.8 \pm 3.9\%$ of control values ($p < 0.05$, $n = 11$; **Figure 6A**). This decrease in amplitude was accompanied by a significant increase in PPR from 1.02 ± 0.0 to 1.16 ± 0.0 ($p < 0.05$). In four of the cells in which baclofen effects on Lf-IPSPs were observed and fully recovered upon washout, the GABA_B receptor antagonist CGP55845 (200 nM) was then applied. Preincubation of CGP55845 alone had no effect on PPR ($p > 0.1$) or IPSP amplitude ($p > 0.1$). No significant changes were observed after further addition of baclofen (**Figure 6B**). These results together indicate a presynaptic depression on Lf-IPSPs due to activation of GABA_B autoreceptors. In the CAA pathway, baclofen decreased the peak amplitudes of the first and second CAA-IPSPs to $70.3 \pm 5.2\%$ and $82.9 \pm 9.4\%$ (**Figure 7A**; $p < 0.05$) of control values, respectively with an increase in PPR (from 1.05 ± 0.1 to 1.28 ± 0.1 , $p < 0.05$). The antagonist CGP55845 (200 nM) was used for incubation in four cells after recovery from the first application of baclofen. Bath application of CGP55845 alone had no significant effect on the amplitude or PPR of CAA-IPSPs. This preincubation antagonized the baclofen induced reduction in amplitudes and PPR facilitation ($n = 4$, $p > 0.05$; **Figure 7B**).

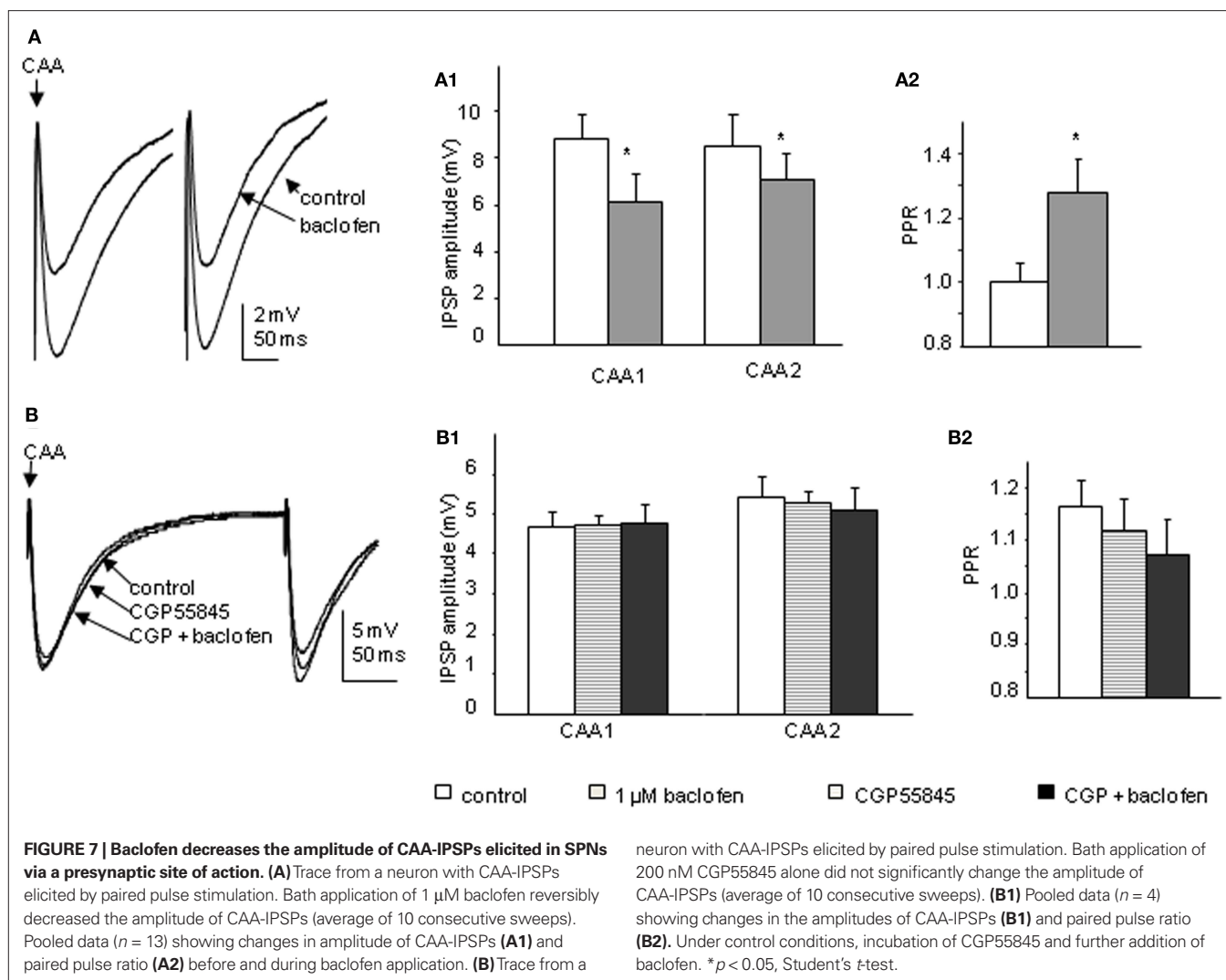
The sensitivity of evoked IPSPs to GABA_A receptor antagonists was examined after blocking polysynaptic transmission and ionotropic glutamate receptors by kynurenic acid. Consistent with previous studies (Deuchars et al., 1997, 2005; Brooke et al., 2004), both Lf- and CAA-IPSPs were reduced in amplitude after bath application of $10 \mu\text{M}$ bicuculline (data not shown). The sensitivity to a GABA_A receptor antagonist suggests the nature of these IPSPs to be GABAergic.

Spontaneous IPSPs were observed in two SPNs recorded. Bath application of $1 \mu\text{M}$ baclofen dramatically reduced the frequency of sIPSPs in both cells, leaving only a few detectable events (**Figure 8**). Further detailed analysis was not performed since the significant drug effect did not leave enough events to be compared. However, the spectacular decrease in the number of IPSPs also signifies a presynaptic site of action.

DISCUSSION

There are two main observations from this study; firstly that activation of presynaptic GABA_B autoreceptors caused attenuation of inhibitory postsynaptic potentials elicited by both Lf and CAA pathways. This suggests that there are GABA_B autoreceptors located on





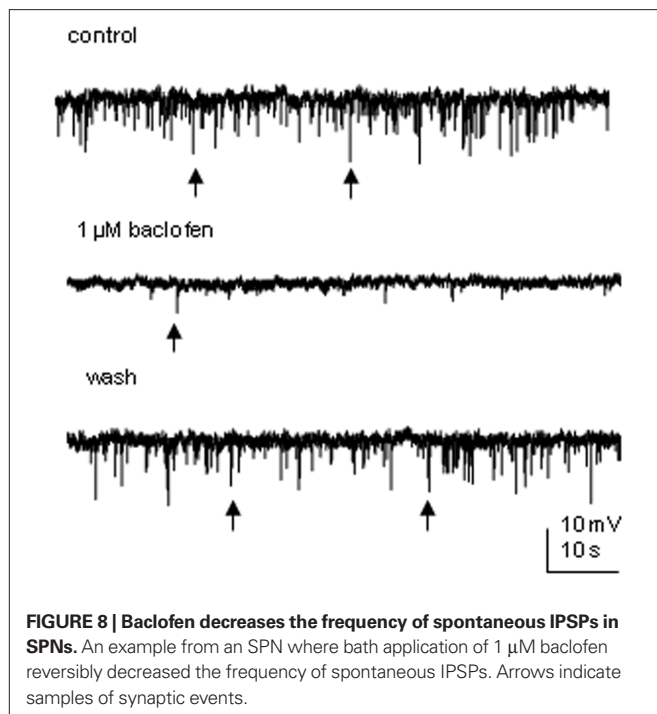
terminals of both descending and local neurons influencing SPN activity. Secondly, very low concentrations of baclofen induced a number of responses in SPNs and also hyperpolarized interneurons—differences in responses may be due to the second messenger systems mediating these responses.

PRESYNAPTIC MODULATION BY GABA_B AUTORECEPTORS

Bath application of the GABA_B receptor agonist baclofen decreased the amplitudes of evoked IPSPs in SPNs elicited from both GABAergic pathways. The reduction was accompanied by an increased PPR which indicates a presynaptic site of action, mediated through an inhibition of GABA release. Preincubation of the antagonist CGP55845 attenuated the agonist induced effects. The likelihood that this was a presynaptic action was strengthened by the observation that the frequency of spontaneous IPSPs, where observed, was also reduced by baclofen.

The concept of GABA_B receptors serving as autoreceptors located at the GABAergic terminals in the CNS is well known. When activated, GABA_B receptors cause inhibition of high voltage sensitive calcium channels (Mintz and Bean, 1993; Poncer et al., 1997) or can have a direct effect on synaptic

vesicle priming (Sakaba and Neher, 2003) thus reducing neurotransmitter release. In our study, IPSPs elicited from Lf and CAA were affected to a similar degree by both the agonist baclofen (at low concentrations) and a broad spectrum antagonist CGP55845, indicating that the regulation of the two pathways is mediated through GABA_B receptors with comparable pharmacological properties. GABA_B receptors are composed of two subunits (GABA_{B1} and GABA_{B2}) that form functional heterodimers (Marshall et al., 1999) and GABA_{B1} subunits can be further divided into GABA_{B1a} and GABA_{B1b} (Kaupmann et al., 1997; Kaupmann et al., 1998). In fact evidence suggests that presynaptic GABA_B receptors are mainly comprised of GABA_{B1a}/GABA_{B2} subunits (Billinton et al., 1999; Towers et al., 2000), so pharmacological differences would be unexpected. Hippocampal studies have reported that presynaptic GABA_B receptors with similar properties are located on both stratum radiatum and stratum oriens interneurons, whilst mGluR receptors are limited to synaptic inputs from stratum oriens interneurons (Poncer et al., 2000). This further supports the idea that presynaptic GABA_B receptors are quite homogenous. Stimulating within the CAA activates GABAergic interneurons



located within this region (Deuchars et al., 2005) and this area receives dense descending inputs from the medial prefrontal cortex. This region of the cortex is a defined vasomotor center, contributing a hypotensive influence (Bacon and Smith, 1993) and this effect may be manifested through direct activation of these local inhibitory GABAergic interneurons in the spinal cord that consequently inhibit SPNs. LF stimulation activates direct descending inhibitory pathways from regions such as the rostral ventrolateral medulla (Miura et al., 1994; Deuchars et al., 1997). Activation of presynaptic GABA_B receptors will reduce both the descending and local inhibitory influences on sympathetic outflow, regardless of their source or their target SPN. It seems that the heterogeneity is observed with the postsynaptic, rather than the presynaptic receptors onto SPN.

PRESYNAPTIC GABA_B RECEPTORS ARE NOT TONICALLY ACTIVATED

The possibility that autoreceptors can be tonically activated at GABAergic terminals onto SPNs was examined in this study. Preincubation of GABA_B receptor antagonist CGP55845 had no significant effect on evoked IPSPs from both pathways. Thus, the autoreceptors located at the GABAergic terminals innervating SPNs are unlikely to be stimulated by ambient GABA, even after synaptic release. Physiological stimulation of GABA_B receptors seems to require strong synaptic stimulation which suggests that high GABA releases are required to reach and thus stimulate the receptors (Isaacson et al., 1993; Scanziani, 2000). This is likely due to the efficient uptake systems in place to remove GABA since uptake inhibitors increase the likelihood of activation of the GABA_B receptors (Isaacson et al., 1993; Scanziani, 2000). The absence of tonic GABA_B activation on SPNs has also been reported using a splanchnic nerve–spinal cord preparation (Cheng et al., 2005).

DISTINCT POSTSYNAPTIC RESPONSES TO LOW CONCENTRATIONS OF BACLOFEN IN SPNs

Postsynaptic GABA_B receptors are predominantly coupled to G protein coupled inward rectifying potassium channels (GIRKs) and therefore cause an inhibitory effect on the cell by increasing K⁺ conductance. However, not all SPNs exhibited a typical large response after challenge with low concentrations of baclofen. Indeed, many exhibited much smaller responses that were not accompanied by changes in input resistance. One possible explanation for the more moderate responses observed in some SPNs is that the SPN itself is not affected directly by GABA_B receptors located on its postsynaptic membrane but that the hyperpolarization is mediated by an effect on neighboring SPNs that are coupled by gap junctions to the recorded neuron and this hyperpolarization is relayed through the gap junctions. However, in non-responding neurons, spikelets were also observed that were reduced or abolished by application of baclofen with no accompanying change in membrane potential. This therefore makes the theory of the moderate response being due to conduction of hyperpolarizing responses from neighboring neurons less likely, although it cannot be totally discounted since some spikelets do remain.

Our data demonstrate that these moderate hyperpolarizations to low concentrations of baclofen were observed in some SPNs whether recordings were made either in K⁺ based or Cs-based intracellular solution. The moderate response is likely to be inhibition of voltage gated calcium channels since nominally Ca²⁺ free solution or cadmium further reduced this effect. This is similar to responses observed by (Cheng et al., 2005), who used higher concentrations of baclofen. We cannot rule out the possibility that the Ca²⁺ activated K⁺ channels contribute to the moderate response observed since these may not be blocked completely with the potassium blockers used, especially in more distal regions of the neuron where complete dialysis of the neuronal content is unlikely to be achieved. However in support of a role of calcium channels, recent studies (Vigot et al., 2006; Perez-Garci et al., 2006) using knockout mice (either 1b^{-/-} or 1a^{-/-}) concluded that activation of GABA_{B1(b)} containing postsynaptic receptors directly blocks dendritic Ca²⁺ channels of layer 5 of cortical pyramidal neurons.

The distinct responses may reflect activation of different postsynaptic receptor subtypes or association with distinct second messenger systems. Such a suggestion is supported by data from substantia nigral cells that showed GABA_B receptors linked to different GIRK channels (Koyrakh et al., 2005). Multiple subtypes of GABA_B receptors have been suggested after observing the effects of different selective antagonists (Solis and Nicoll, 1992; Cunningham and Enna, 1996). Moreover, two types of pharmacologically distinct receptors were used to explain the residual responses of thalamocortical neurons mediated by baclofen (Guyon and Leresche, 1995). In fact evidence suggests that it is the GABA_{B1a/2} complex that exhibits the greater sensitivity to low concentrations of baclofen in the hippocampus that may be due mainly to differences in the density of those receptors over GABA_{B1b/2} complexes, rather than differences in their affinity (Guettg et al., 2009). Thus hyperpolarizations observed during application of 1 μM baclofen in some cells could be mediated through preferential activation of receptors present in a higher density. There are developmental changes in GABA_B receptor subunits since GABA_{B1(a)} is

predominant during embryonic period and at birth whilst GABA_{BI(b)} expression increases during the first postnatal month in the brain (Fritschy et al., 1994). Our PCR results show that all three subunits are in fact present at the age that we carried out the experiments, so absence of a subunit may not be a contributing factor to our findings. A detailed study of receptor subunit composition and sub-cellular localization would provide first hand information to understand whether they are related to the pharmacological distinctions observed. For example, GABA_{BI(b)} subunit containing receptors have been reported to be present extrasynaptically in cerebellar Purkinje cells (Fritschy et al., 1999), dorsal cochlear nucleus (Lujan et al., 2004) and hippocampal CA1 pyramidal neurons (Kulik et al., 2006).

FUNCTIONAL SIGNIFICANCE AND CONCLUSION

The results of activation of GABA_B receptors in sympathetic regulation are complicated and sometimes contradictory. Due to the multiple functions of GABA_B receptors, it is not surprising that different experimental protocols lead to completely different conclusions. For example, intrathecal injection of baclofen in the thoracic spinal cord decreases cardiac sympathetic activity (Kim et al., 2000) whilst intracerebroventricular or intraperitoneal administration of baclofen leads to an increase in blood pressure and heart rate (Persson and Henning, 1980). In a spinal cord–splanchnic nerve preparation, bath application of baclofen suppresses splanchnic nerve activities possibly via two mechanisms: direct inhibition of membrane excitability by affecting K⁺ or Ca²⁺ currents and/or attenuation of excitatory synaptic events to SPNs (Cheng et al., 2005).

In this study, the effects of stimulation of postsynaptic receptors and presynaptic autoreceptors in SPNs were studied. Although pharmacologically distinct subtypes of postsynaptic GABA_B

receptors have not been proven, the fact that hyperpolarization was only observed in some SPNs (and possibly all interneurons) of the IML when using low concentrations of agonist might suggest a physiological significance underlying these differences in sensitivity. Indeed many previous studies examining characteristics of SPNs have reported differences in spontaneous activity (Spanswick and Logan, 1990), electrical coupling (Logan et al., 1996), other intrinsic membrane properties (Zimmerman and Hochman, 2010) and the synaptic inputs that they receive (Deuchars et al., 1997; Deuchars et al., 1995), that may reflect functional subgrouping of SPNs. Our study further supports this idea with distinct groups of SPN perhaps possessing specific GABA_B receptors and suggests that such a difference may be a useful pharmacological target in the future. Presynaptic GABA_B receptors regulating the sympathetic outflow at the spinal levels have been previously reported but mainly focused on its action at the excitatory nerve terminals (McKenna and Schramm, 1984; Wu and Dun, 1992). This study provides evidence that activation of GABA_B autoreceptors suppresses GABAergic inhibitory transmission. The regulation of two distinct GABAergic pathways shows similar characteristics. These results somewhat improve the current knowledge on the function of GABA_B receptors in sympathetic control and lend strength to the idea that these autoreceptors at least would not present a suitable target for differential control of the two pathways.

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