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Inflammatory mediators potentiate high affinity GABA_A currents in rat dorsal root ganglion neurons

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Abstract

Following acute tissue injury action potentials may be initiated in afferent processes terminating in the dorsal horn of the spinal cord that are propagated back out to the periphery, a process referred to as a dorsal root reflex (DRR). The DRR is dependent on the activation of GABA_A receptors. The prevailing hypothesis is that DRR is due to a depolarizing shift in the chloride equilibrium potential (E_{Cl}) following an injury-induced activation of the Na⁺-K⁺-Cl⁻-cotransporter. Because inflammatory mediators (IM), such as prostaglandin E₂ are also released in the spinal cord following tissue injury, as well as evidence that E_{Cl} is already depolarized in primary afferents, an alternative hypothesis is that an IM-induced increase in GABA_A receptor mediated current (I_{GABA}) could underlie the injury-induced increase in DRR. To test this hypothesis, we explored the impact of IM (prostaglandin E₂ (1 μM), bradykinin (10 μM), and histamine (1 μM)) on I_{GABA} in dissociated rat dorsal root ganglion (DRG) neurons with standard whole cell patch clamp techniques. IM potentiated I_{GABA} in a subpopulation of medium to large diameter capsaicin insensitive DRG neurons. This effect was dependent on the concentration of GABA, manifest only at low concentrations (< 10 μM). THIP evoked current were also potentiated by IM and GABA (1 μM) induced tonic currents enhanced by IM were resistant to gabazine (20 μM). The present data are consistent with the hypothesis that an acute increase in I_{GABA} contributes to the emergence of injury-induced DRR.

Keywords

primary afferent; nociceptor; inflammation; voltage-clamp; excitation-inhibition balance

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Introduction

It has long been appreciated that afferent volleys into the spinal cord are followed by a depolarization of the central afferent terminals, a phenomenon referred to as primary afferent depolarization (PAD) [7]. Subsequent data indicated that PAD is due to GABA_A receptor activation (for review [23, 29]), that is associated with membrane depolarization in primary afferent neurons because, unlike neurons in the central nervous system, the Cl⁻ equilibrium potential (E_{Cl}) in primary afferents is maintained at a relatively elevated level into adulthood [2, 25]. The elevated E_{Cl} appears to be due to the fact the Na⁺-K⁺-Cl⁻-cotransporter, NKCC1 expression is maintained in sensory neurons with no compensatory increase in the K⁺-Cl⁻-cotransporter, KCC2 [20].

Following tissue injury or intense noxious stimulation, afferent volleys that would normally result in a PAD can actually initiate antidromically propagated action potentials, a phenomenon referred to as a dorsal root reflexes (DRR) [29]. The DRR is also dependent on GABA_A receptor activation [21] and is blocked by the NKCC1 antagonist bumetanide [27]. Furthermore, tissue injury is associated with first an increase in the phosphorylation of NKCC1 and then an increase in the translocation of this co-transporter to the membrane [10]. It has therefore been hypothesized that the emergence of the DRR is due to an increase in NKCC1 activity resulting in a depolarizing shift in E_{Cl} [5, 19]. The shift in E_{Cl} would enable the GABA-induced depolarization to drive the membrane potential above the action potential threshold, resulting in a DRR.

Recent evidence, however, suggests that E_{Cl} for many sensory neurons is already above the action potential threshold [22]. Furthermore, there is evidence that inflammatory mediators (IM) such as prostaglandin E₂ are released in the spinal cord following noxious stimulation [18], as well as evidence that GABA_A receptors may be dynamically regulated via an increase in kinase activity [14]. Together, these observations suggested an alternative to the NKCC1 hypothesis, which is that the emergence of the DRR is due to an IM-induced increase in GABA_A current. The present study was designed to test this hypothesis. Isolated DRG neurons were studied with whole cell patch clamp techniques to record GABA_A currents before and after the application of a combination of inflammatory mediators.

Methods

Adult (180-280g) male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used for all experiments which were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines as well as guidelines established by the International Association of the Study of Pain for the use of laboratory animals in research. L4-5 DRG were harvested from the rats deeply anesthetize with a cocktail containing ketamine (55mg/kg), xylazine (5.5 mg/kg), and acepromazine (1.1mg/kg), enzymatically treated, mechanically dissociated, and plated on laminin and ornithine-coated (Life Technologies, Grand Island, NY) cover-slips as previously described [17]. After plating for two hours, cells were flooded with Minimal Essential Media (MEM) containing 10% fetal bovine serum, 1000 units of penicillin-streptomycin, and 1x MEM vitamins (Life Technologies) and were placed in a CO₂ (3%) incubator at 37°C for 24 hours prior to study.

Cultured DRG neurons were used in the present experiment because 1) we wished to be able to detect the impact of IM on both low and high affinity GABA receptors and 2) recent data indicates that there is an increase in high affinity GABA receptors in DRG neurons with time in culture [15].

Whole-cell patch-clamp recordings were performed with a HEKA EPC10 amplifier (HEKA Elektronik GmbH, Lambrecht, Germany). Data were acquired at 10 kHz and filtered at 2 kHz unless otherwise noted. Borosilicate glass (WPI, Sarasota, FL) electrodes were 2–3 M Ω when filled with the following solution (mM): CsCl 140, MgCl₂ 1, EGTA 11, HEPES 10, Mg-ATP 2, and GTP 1; pH was adjusted to 7.2 with Tris-base and osmolality was adjusted to 310 mOsm with sucrose. The bath solution contained the following (in mM): NaCl 130, KCl 3, CaCl₂ 2.5, MgCl₂ 0.6, HEPES 10, and glucose 10; pH was adjusted to 7.4 with Tris-base and osmolality was adjusted to 320 mOsm with sucrose. All reagents used for the preparation of recording solutions were obtained from Sigma-Aldrich (St Louis MO).

Neurons were held at –60 mV. Currents were evoked by 3 or 60 seconds of focal application of GABA or GABA_A receptor agonists. After establishing whole cell access and compensating capacitive transients with amplifier circuitry, a stable baseline evoked current was established with at least a 3min inter-test-interval. Following establishment of stable GABA or THIP evoked currents, current amplitude was determined during and after 5 min application of IM. Capsaicin (500 nM) was applied at the end of experiment to help identify the phenotype of the neuron studied. A neuron was considered responsive to capsaicin if current evoked was greater than 3 times the maximal deflection in baseline observed with vehicle application.

A local superfusion system (Fast Step, Warner Instruments, Hamden CT) was used for drug delivery. Drugs were diluted to final concentrations in bath solution from stock solutions at least 1000 times greater than the highest concentration employed. Drugs used were GABA, δ subunit containing GABA_A receptor agonist (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP) and allosteric modulator tetrahydrodeoxycorticosterone (THDOC), and GABA_A receptor antagonists gabazine (SR95531) and bicuculline. Inflammatory mediators consisted of a combination of histamine (1 μ M, dissolved in water, 100mM stock concentration), bradykinin (10 μ M, dissolved in 1% acetic acid, 23.58 mM stock concentration), and prostaglandin E₂ (1 μ M, dissolved in 100% ethanol, 10 mM stock concentration). All drugs were obtained from Sigma-Aldrich and prepared as stock solution stored –20 then diluted in bath solution before use.

Data are expressed as mean $\pm$ SEM. Differences between groups were assessed with a one-way ANOVA or a Students t-test, where $p < 0.05$ was considered statistically significant.

Results

In initial experiments with GABA concentrations $>60 \mu$ M ($n = 12$), there was no detectable change in I_{GABA} in the presence of IM. However, in 7 neurons tested with concentrations of GABA $< 10 \mu$ M, there was an increase in I_{GABA} in the presence of IM. This suggested the impact of IM was dependent on the concentration of GABA used. To address this possibility, GABA concentration-response data were collected before, and during a 5 minute superfusion with IM. In 6 of 22 neurons tested, current evoked by 1 and 3 μ M of GABA was significantly increased in the presence of IM from 2.5 ± 1.1 pA/pF to 9.9 ± 2.9 pA/pF ($p < 0.01$), and from 5.2 ± 1.7 pA/pF to 9.6 ± 1.2 pA/pF ($p < 0.05$), respectively. Interestingly, a response (3.19 ± 1.59 pA/pF) to a previously ineffective concentration of GABA (0.3 μ M) was detected when it was applied with IM. In contrast, there was no IM-induced increase in current at concentrations of GABA 10 μ M (Fig 1B) or higher (Fig. 1C). This increase in current persisted following wash of IM, such that I_{GABA} was still 89 ± 16.8 % greater than baseline at 5 minutes after IM wash. The absence of an increase in total I_{GABA} in combination with an increase in current at low concentrations of GABA suggested IM was increasing the potency of GABA at a subpopulation of presumably high affinity GABA receptors.

In attempt to identify the putative phenotype of the neurons responsive to IM (i.e., in which IM induced an increase in I_{GABA}), we compared cell body size and sensitivity to capsaicin of IM responders and non responders. The average membrane capacitance of the IM responders was 42.2 ± 3.8 pF, while that of non-responders was 25.2 ± 2.1 pF ($p < 0.05$). While 5 of 16 non-responders were responsive to capsaicin (500 nM), none of the 6 responders tested were responsive to capsaicin. These results suggest that IM responders may represent a subpopulation of non-nociceptive afferents.

We recently obtained evidence indicating that δ -subunit containing receptors contribute to the subpopulation of high affinity $GABA_A$ receptors in DRG neurons [15]. The δ -subunit preferring agonist THIP was therefore used to determine whether δ -subunit containing receptors may be the target of IM actions. In a subpopulation of neurons tested (8 of 20), the currents evoked by 30 μ M of THIP were potentiated by IM ($p < 0.05$, $n = 8$, Fig. 2A, B). Neurons responsive to IM were all responsive to THIP at 30 μ M or lower, while those unresponsive to IM were an order of magnitude less sensitive to THIP (Fig 2C). As with the results of the GABA current experiments, membrane capacitance of IM responsive neurons 81.9 ± 3.2 pF was significantly larger than that of neurons unresponsive to IM (28.8 ± 2.0 pF, $p < 0.01$). Furthermore, none of the IM responsive neurons tested with THIP were capsaicin sensitive while more than half of the IM unresponsive neurons tested (11 of 17) were capsaicin sensitive.

Three additional experiments were performed to further test the contribution of δ subunit containing receptors to the current potentiated by IM. Based on the biophysical and pharmacological properties of δ subunit containing receptors [8, 24, 31], we first determined whether the current potentiated by IM exhibited desensitization during prolonged (1 minute) GABA application. Second, we compared the impact of SR-95531 (20 μ M) and bicuculline (50 μ M) on tonic currents potentiated by IM. Finally, we assessed the impact of THDOC on IM potentiated tonic currents. Results of these experiments indicated that IM potentiates a tonic current evoked with 1 μ M GABA that is resistant to SR-95531 (20 μ M) and potentiated by THDOC (Fig 3A). These tonic currents are completely blocked by bicuculline (Fig 3B). To quantify the IM-induced increase in tonic current, we assessed the bicuculline sensitive current before and during application of IM. Pooled data (Fig 3C) indicate that the IM-induced increase in tonic current is also statistically significant ($p < 0.05$, $n = 5$).

Discussion

The present study was designed to test the hypothesis that the emergence of the DRR is due to an IM-induced increase in $GABA_A$ current. Consistent with our hypothesis, IM-induced an increase in I_{GABA} in a subpopulation of neurons studied. This increase persisted after wash of IM and was associated with a partial shift in the GABA concentration response curve rather than an increase in total current. The IM sensitive current had biophysical and pharmacological properties of an extrasynaptic high affinity δ subunit containing receptor [8, 24, 31].

There is evidence that bradykinin activates a Ca^{2+} -dependent Cl^- current in DRG neurons [16] which was recently identified as being carried by TMEM16A, also known as ANO1 [16]. There is also evidence that inflammatory mediators, including bradykinin can drive an increase in intracellular Ca^{2+} in sensory neurons [28]. However, we have no evidence that this current contributed to actions of IM in the present study. That is, the only IM-induced increase in current observed, was observed in the presence of GABA, with no detectable increase in holding current in the absence of GABA. Furthermore, both the GABA evoked current and IM potentiated current were completely blocked by the $GABA_A$ receptor

antagonist, bicuculline. Why we were not able to detect an IM-induced increase in Ca^{2+} -dependent Cl^- current in the present study is not entirely clear, but may be due to the fact that this outwardly rectifying current is minimized at -60 mV, the holding potential used for the study of GABA currents in the present experiments.

The selective influence of IM on putative non-nociceptive afferents was striking for several reasons. First, we have evidence that δ -subunit containing currents are dramatically increased under the culture conditions used in the present study, even in putative nociceptive afferents [15]. Second, it has been demonstrated that IM can drive the modulation of a number of ion channels in putative nociceptive DRG neurons (for review [12]), indicating that receptors for these mediators are both present and functional in putative nociceptive DRG neurons in culture. Third, the focus of prior studies of injury-induced changes in GABA_A signaling [3], was to identify mechanisms that enable GABA to contribute to the increase in pain associated with acute tissue injury and/or inflammation. Thus, we anticipated that the inflammatory mediator-induced increase in GABA_A current would be manifest in putative nociceptive afferents.

Because there is evidence for a subpopulation of nociceptive afferents with rapidly conducting axons [30], it is possible that the subpopulation of IM sensitive afferents observed in the present study are nociceptive and that the IM-induced increase in GABA_A currents contributes to emergence of a pronociceptive role for GABA following tissue injury. However, if, as suggested by their phenotype, this subpopulation is non-nociceptive, the functional significance of an IM-induced increase in GABA current in this subpopulation is more complicated. On one hand, non-nociceptive afferents have TTX-sensitive Na^+ currents subject to steady-state inactivation. An increase in GABA_A mediated current in these neurons would result in a membrane depolarization-induced inactivation of channels necessary for action potential propagation, and thus, the inhibition of non-nociceptive input. This may, in turn, result in the facilitation of nociceptive input normally inhibited by activity in non-nociceptive afferents [11]. On the other hand, GABA_A mediated depolarization of these neurons may be sufficient to facilitate low threshold afferent input to the superficial dorsal horn [4] which may attenuate nociceptive input into the dorsal horn. Additional studies would be needed to distinguish between these possibilities.

The mechanism(s) underlying the action of IM remain to be elucidated. However, in contrast to the increase in GABA potency at receptors already present on the membrane suggested by the results of the present study, an increase in receptor density has been far more commonly reported. The increase in receptor density appears to be due to changes in receptor trafficking, which has been shown to be mediated by tyrosine kinase [13], protein kinase C [1], and calcium calmodulin-dependent protein kinase II [6]. Thus, available evidence argues against second messenger pathways engaging these kinases as the mechanisms underlying the actions of IM in the present study. There is evidence from retina amacrine cells that protein kinase A-dependent (PKA) phosphorylation of GABA_A receptors results in an increase in GABA potency with no change in efficacy [9], as was observed in the present study, although the GABA_A receptors present in amacrine cells appear to have a relatively low affinity for GABA (i.e., ~ 70 μM). That said, it has long been appreciated that PKA is a major signaling pathway underlying the actions of inflammatory mediators including PGE_2 [26].

Conclusions

The IM-induced potentiation of high affinity tonic GABA_A currents in a subpopulation of putative non-nociceptive afferents suggests that the shift in the balance of nociceptive and non-nociceptive input to the CNS associated with tissue injury may be associated with

changes in non-nociceptive input in addition to the well described increase in nociceptive input. This observation also suggests that there is subcellular compartmentalization of second messenger signaling cascades in primary afferents that it retained even in the isolated afferent cell body. Finally, given preliminary evidence that persistent inflammation is associated with an increase in GABA_A current that is not due to a shift in GABA potency, but an increase in GABA functional receptors on the membrane [32], the acute IM-induced modulation of GABA currents suggest that there are time dependent changes in the regulation of GABA_A receptors in sensory neurons following the induction of inflammation.

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Abbreviations

DRG	dorsal root ganglion
DRR	dorsal root reflex
E_{Cl}	Cl ⁻ equilibrium potential
GABA	γ-aminobutyric acid
I_{GABA}	GABA _A current
IM	inflammatory mediators
KCC2	K ⁺ -Cl ⁻ -cotransporter
NKCC1	Na ⁺ -K ⁺ -Cl ⁻ -cotransporter
PAD	primary afferent depolarization
SR-95531	gabazine
THDOC	tetrahydrodeoxycorticosterone
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol

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Highlights

GABAA excites primary afferents in the presence of inflammation.

GABAA receptors are dynamically regulated in neurons in the central nervous system.

Inflammatory mediators induced an increase in GABA potency at high affinity receptors.

This is the first demonstration of the dynamic regulation of GABA_A currents in primary afferents.

Figure 1.

Impact of inflammatory mediators (IM) on GABA-evoked currents (I_{GABA}). Representative examples of I_{GABA} evoked before (gray traces) and after (black traces) IM application in response to 1 μM (A) and 10 μM (B) GABA, respectively. GABA and IM application are indicated by solid and dashed bars, respectively. C: Concentration-response relationships for GABA before (open circle) and 5 minutes after (filled circle) IM application ($n = 6$). Data from each neuron were normalized with respect to maximal inward current (I_{max}). * is $p < 0.05$ and ** is $p < 0.01$.

Figure 2.

Impact of inflammatory mediators (IM) on THIP evoked currents (I_{THIP}). **A:** Representative example of current evoked by 30 μM of THIP in a DRG neuron with a large cell body diameter (65 pF) that was capsaicin insensitive before (gray trace) and 5 minutes after (black trace) IM application. Solid and dashed bars indicate THIP and IM application, respectively. **B:** Concentration-response relationships for THIP before (open circle) and 5 minutes after (filled circle) IM application in medium to large diameter (>45 pF) capsaicin insensitive neurons (n = 8). **C:** There was no detectable influence of IM on THIP evoked currents in small diameter neurons at least half of which were capsaicin sensitive (n=17). * is $p < 0.05$.

Figure 3.

Pharmacology of inflammatory mediator (IM) potentiated high affinity tonic GABAA current in DRG neurons. **A:** Tonic GABA current was evoked with a prolonged application of GABA (1 μM, gray bar). This current was not blocked by SR95531 (20 μM), but was potentiated by THDOC (0.1 μM) which were applied as indicated by black horizontal bars.. **B:** Tonic currents evoked with 1 μM GABA were blocked by bicuculline (50 μM) whether (black trace) or not (gray trace) currents were potentiated by IM. Dotted lines in A and B indicate 0 current. **C:** Pooled data from 8 DRG neurons with a membrane capacitance 45 pF, in which the bicuculline sensitive current was used to quantify the magnitude of the tonic current evoked before (control) and after (IM) the application of IM. * is $p < 0.05$