Evaluation of the Effect of $GABA_B$ Agonists on the Vagal Nodose C-Fibers in the Esophagus

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Summary

Clinical studies showed that $GABA_B$ receptor agonists improve symptoms in patients with gastroesophageal reflux disease. One proposed mechanism of this effect is direct inhibition of the gastroesophageal vagal tension mechanosensors by $GABA_B$ agonists leading to reduction of reflux. In addition to tension mechanosensors, the vagal nodose ganglion supplies the esophagus with nociceptive C-fibers that likely contribute to impairment of esophageal reflex regulation in diseases. We hypothesized that $GABA_B$ agonists inhibit mechanically-induced activation of vagal esophageal nodose C-fibers in baseline and/or in sensitized state induced by inflammatory mediators. *Ex vivo* extracellular recordings were made from the esophageal nodose C-fibers in the isolated vagally-innervated guinea pig esophagus. We found that the selective $GABA_B$ agonist baclofen (100-300 μM) did not inhibit activation of esophageal nodose C-fibers evoked by esophageal distention (10-60 mmHg). The mechanical response of esophageal nodose C-fibers can be sensitized by different pathways including the stimulation of the histamine H_1 receptor and the stimulation the adenosine A_{2A} receptor. Baclofen failed to inhibit mechanical sensitization of esophageal nodose C-fibers induced by histamine (100 μM) or the selective adenosine A_{2A} receptor agonist CGS21680 (3 nM). Our data suggest that the direct mechanical inhibition of nodose C-fibers in the esophagus is unlikely to contribute to beneficial effects of $GABA_B$ agonists in patients with esophageal diseases.

Key words

Esophagus • Vagal nodose C-fibers • Extracellular nerve recording • $GABA_B$ agonists • Baclofen

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Introduction

Several clinical studies have shown that the γ-aminobutyric acid type B ($GABA_B$) receptor agonists including baclofen have potential to improve the symptoms in patients with gastroesophageal reflux disease (Lehmann *et al*. 1999, Koek *et al.* 2003, Vela *et al*. 2003, Lehmann 2009, Boeckxstaens *et al*. 2010). The main proposed mechanism of this effect is the reduction of reflux due to direct peripheral inhibition of the gastroesophageal vagal tension mechanosensors by $GABA_B$ agonists (Blackshaw *et al.* 1999, Lehmann *et al.* 1999, Liu *et al.* 2002, Zagorodnyuk *et al.* 2002, Lehmann 2009, Boeckxstaens 2011). It has been postulated in these studies that the inhibition of tension mechanosensors inhibits the reflex triggering of transient lower esophageal sphincter relaxations (TLESR) and thereby reducing the reflux (Lehmann *et al*. 1999, McDermott *et al*. 2001, Zhang *et al*. 2002, Koek *et al.* 2003, Lehmann 2009, Boeckxstaens *et al*. 2010).

Vagal tension mechanosensors are considered the key afferent nerve subtypes regulating the motility of gastrointestinal organs under normal circumstances. Similar to other species the vagal tension mechanosensors in the guinea pig esophagus originate from the vagal afferent nodose ganglia and are exquisitely sensitive to esophageal distention (Page and Blackshaw 1998, Zagorodnyuk and Brookes 2000, Blackshaw *et al.* 2000, Yu *et al.* 2005). Esophageal tension mechanosensors in the guinea pig are neurophysiologically A-fibers and are

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unresponsive to the nociceptive activator capsaicin, and other noxious stimuli (Kollarik *et al.* 2010). However, in addition to A-fiber tension mechanosensors the vagal nodose ganglia also project a large population of C-fibers into the esophagus (Yu *et al.* 2005). Unlike tension mechanosensors, these nodose C-fibers have higher threshold for esophageal distention, and are sensitive to capsaicin and other potentially noxious stimuli (Kollarik *et al.* 2010). It is very likely that the activation of these prototypical nociceptive nodose C-fibers contributes to reflex dysregulation of esophageal function in pathological circumstances such in gastroesophageal reflux disease (GERD).

We therefore hypothesized that the $GABA_B$ agonists modulate the activity of esophageal nodose C-fibers. We evaluated the effect of $GABA_B$ agonists on the mechanical response to esophageal distention that is the most relevant activator of nodose C-fibers and triggers esophageal motor reflexes. We investigated the effect in both the naïve state and in sensitized states induced by relevant esophageal pro-inflammatory stimuli. Nonetheless, we found that the $GABA_B$ agonists failed to modulate the activity of the esophageal nodose C-fibers.

Material and Methods

Male Dunkin Hartley guinea pigs (Department of Experimental Pharmacology, Slovak Academy of Science, Dobra Voda, Slovakia) weighing 200-250 g were used. All experiments were approved by the Jessenius Faculty of Medicine Ethic Committee in accordance with applicable laws and policies.

Extracellular recordings from vagal nodose nociceptors

Extracellular recordings from vagal neurons were described previously (Yu *et al.* 2005). Extracellular recordings were made from vagal nodose neurons with mechanosensitive nerve terminals in the esophagus in an isolated, perfused, vagally innervated guinea pig esophagus preparation. The esophagus and trachea were dissected with preserved bilateral extrinsic vagal innervation (including nodose ganglia). The tissue was pinned in a small Sylgard-lined Perspex chamber filled with indomethacin (3 μM) containing Krebs solution (in mM: 118 NaCl, 5.4 KCl, 1 NaH_2PO_4, 1.2 $MgSO_4$, 1.9 $CaCl_2$, 25 $NaHCO_3$, and 11 dextrose, gassed with 95 % O_2 – 5 % CO_2, pH=7.4, 35 °C). The esophagus with trachea was pinned in the tissue compartment, and the rostral aspect of the vagus nerves, including the nodose ganglion, were pinned in the recording compartment. The two compartments were separately superfused with Krebs solution (pH=7.4, 35 °C, 4-6 ml/min). Polyethylene tubing was inserted 3-5 mm in the cranial and caudal esophagus and secured for perfusion. The pressure in the fluid (Krebs)-filled esophagus was measured with a differential pressure transducer connected in series to the esophagus and recorded simultaneously with neural activity by the chart recorder (TA240S; Gould, Valley View, OH). Isobaric esophageal distension for 20 s with an intraluminal pressure of 10-30-60 mmHg (generated by a gravity-driven pressure system) separated by 3 min was used to determine the distension pressure-nerve activity relationship of an esophageal afferent fiber. An aluminosilicate glass microelectrode was filled with 3 M sodium chloride (electrode resistance 2 MΩ). The electrode was placed in an electrode holder connected directly to the head stage (A-M Systems). A return electrode of silver-silver chloride wire and earthed silver-silver chloride pellet was placed in the perfusion fluid of the recording compartment. The recorded signal was amplified (M1800; A-M Systems) and filtered (low cut-off, 0.3 kHz; high cut-off, 1 kHz), and the resultant activity was displayed on an oscilloscope (TDS340; Tektronix) and the chart recorder. The data were stored and analyzed on an Apple computer using the software TheNerveOfIt (sampling frequency 33 kHz; PHOCIS, Baltimore, MD). The recording electrode was micromanipulated into the nodose ganglion, and a distension-sensitive unit was identified when esophageal distension (at 60 mmHg for 5 s) evoked action potential discharge.

The chemicals diluted in Krebs solution were delivered in the external perfusion. The nerve activity was monitored continuously and analyzed in 1-s bins (Hz).

Drugs and chemicals

The following drugs were used: adenosine A_{2A} agonist CGS21680 (0.003 μM, Tocris Bioscience, stock sol: 10 mM dissolved in dimethylsulfoxide); histamine (100 μM, Sigma-Aldrich, stock sol: 100 mM dissolved in distilled water); $GABA_B$ agonist – baclofen (100 or 300 μM, Sigma-Aldrich, stock sol: 0.2 M dissolved in 1 N HCl); other $GABA_B$ agonist – SKF97541 (3 μM, Tocris Bioscience, stock sol: 100 mM dissolved in distilled water); TRPV1 agonist – capsaicin (1 μM, Sigma-Aldrich, stock sol: 10 mM dissolved in ethanol). Stock solutions were stored at −20 °C. All drugs were

further diluted in Krebs buffer to indicate final concentrations shortly before use.

Experimental protocol

The chemicals (alone or in combination) diluted in KBS solution were delivered to the esophagus in the external perfusion each for 30 min. The nerve activity (action potential discharge) was monitored continuously and analyzed in 1-s bins. After each superfusion with indicated chemicals isobaric esophageal distension for 20 s with an intraluminal pressure of 10-30-60 mmHg separated by 3 min was used to determine the distension pressure-nerve activity relationship of esophageal afferent fibers.

The effect of baclofen (100, 300 μM) on nodose vagal C-fibers was compared with control mechanical response.

In one series of experiments the distension pressure-nerve activity relationship of esophageal afferent fibers was compared between CGS21680 (0.003 μM) and combination of CGS21680 (0.003 μM) + baclofen (100 μM). In another series of experiments the mechanical response to distention was studied after superfusion to histamine (100 μM) alone and in combination with baclofen (300 μM) + histamine (100 μM). In last series of experiments the effect of SKF97541 (3 μM) alone or in combination with CGS21680 (0.003 μM) + SKF97541 (3 μM) was studied.

Previous experiments have shown that histamine and CGS21680 caused mechanical hypersensitivity in nodose C-fibers (Ru *et al.* 2011, Yu *et al.* 2007). Esophageal sensitization was quantified as the increased response to mechanical stimulation on gradual esophageal distention. The TRPV1 agonist capsaicin was used at the end of each experiment at its maximally effective concentration of 1 μM to confirm capsaicin-positive C-fibers.

Data analysis

The distension-evoked nerve response was quantified as the total number of action potentials within a 20-s distention period presented as means ± SE. For statistical analysis of the change in the overall mechanical response was used the area under the curve (AUC) calculated using standard geometrical formulas with the resultant formula (the units are omitted): AUC = 20 x $(T_{10} + T_{30})/2$ + 30 x $(T_{30} + T_{60})/2$ where T_{10}, T_{30}, and T_{60} are the total number of action potentials at the distention pressures 10, 30, and 60 mmHg, respectively, and the coefficients 20 and 30 refer to difference between tested pressure points used (i.e., 20 mmHg = 30 − 10 mmHg). The AUC was determined in control conditions and following the treatment, and AUC ratio was calculated by dividing AUC following the treatment by AUC in control condition. This normalized ratio allowed for comparison between the groups. In order to evaluate the changes in the time course of action potential discharge the dynamic response index was calculated by dividing the action potential number obtained in the first 3 seconds of the response divided by the action potential number in the first 15 second interval of the response. The dynamic response index was calculated for the response to distention with 30 mmHg that was found most sensitive to the changes in mechanical responsiveness based on the previous publications (Yu *et al.* 2007, Yu and Ouyang 2009). Since the complete recordings were no longer available in few experiments on histamine-induced sensitization, the changes in the time course of action potential discharge were estimated by dividing the peak frequency by the total number of action potentials in 20 s of the distention.

Paired and un-paired *t*-tests were used as appropriate and the significance was defined as $P<0.05$.

Results

First we addressed the hypothesis that the selective $GABA_B$ receptor agonist baclofen inhibits the response of esophageal nodose C-fibers to mechanical stimulation. We used esophageal distention evoked by increased intraesophageal pressure as a relevant mechanical stimulus for the esophageal nodose nociceptive C-fibers. We employed our validated method of distention to 10, 30 and 60 mmHg, each for 20 s (Yu *et al.* 2005). In order to quantitatively describe the mechanical response to esophageal distention in the tested range for the purpose of statistical treatment we used the area under the curve (AUC) (Yu *et al.* 2007). In order to quantify the change of mechanical response we used the AUC ratio – AUC of mechanical response following the treatment divided by AUC of mechanical response prior to the treatment in control conditions. The calculation of AUC and AUC ratio is described in detail in methods.

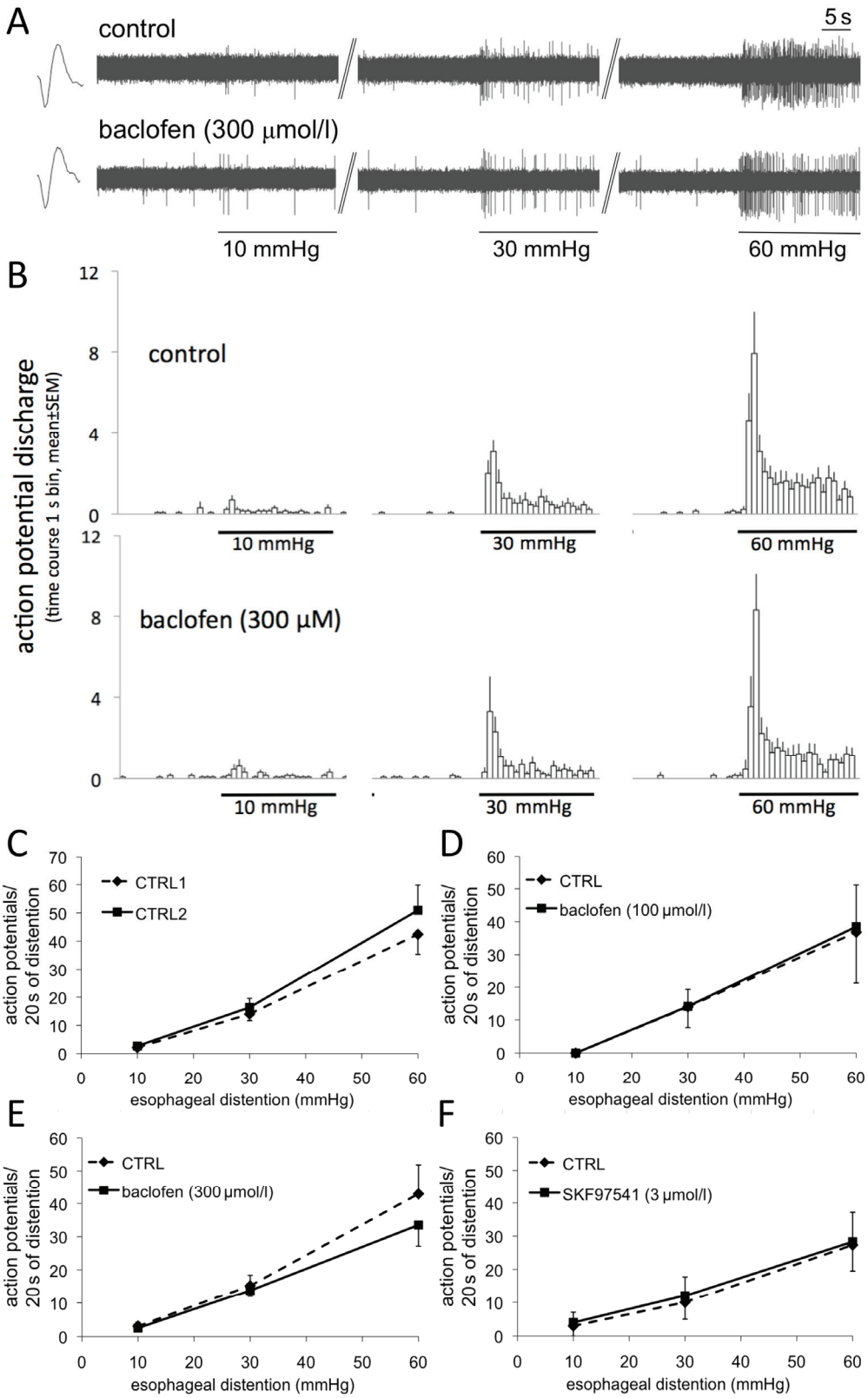

Fig. 1. $GABA_B$ receptor agonists did not inhibit mechanical response of the esophageal nodose C-fibers. (**A**) Representative traces of the extracelullar single nerve fiber recording response of nodose C-fibers to esophageal distention with define pressure before and after superfusion with $GABA_B$ selective agonist baclofen in the concentration of 300 μmol/l. (**B**) The average time course of the action potential discharge evoked by esophageal distention in the absence and presence of baclofen. (**C**) Reproducibility of the response of nodose C-fibers to esophageal distention (n=14). (**D**) The $GABA_B$ selective agonist baclofen in the concentration of 100 μmol/l failed to inhibit mechanical response of nodose C-fibers (n=6). (**E**) Increasing the concentration of baclofen to 300 μmol/l did not reveal the inhibitory effect (n=13). (**F**) Structurally different $GABA_B$ selective agonist SKF97541 also failed to inhibit mechanical response of nodose C-fibers (n=3).

$GABA_B$ receptor agonists did not inhibit mechanical response of the esophageal nodose C-fibers

Initially, we evaluated the reproducibility of mechanical response in our system. In the control conditions we noted a marginal increase of mechanical response when the distention protocol was repeated in 15-30 min intervals (Fig. 1C). Accordingly, the AUC ratio for the repeated mechanical response was 1.2±0.1 (n=14). This was taken into account when evaluating the changes in mechanical response, i.e. the comparisons were made relative to this control experiment.

We first evaluated baclofen in a concentration of 100 μM that was previously found effective to inhibit other types of visceral afferent nerve terminals (Page and Blackshaw 1999). The tissue was incubated with baclofen delivered through superfusion for 30 min. Baclofen (100 μM) did not affect the mechanical response of nodose C-fibers (Fig. 1D). The AUC ratio for the mechanical response in the presence of baclofen (100 μM) was 1.2±0.4 (n=6) that was not different (p=0.8) from AUC for repeated control response (1.2±0.1, n=14, data in Fig. 1C). Increasing the concentration of baclofen to 300 μM did not reveal an inhibitory effect on mechanical response (Fig. 1E). The AUC ratio for the mechanical response in the presence of baclofen (300 μM) was 1.0±0.1 (n=13) that was not different (p=0.2) from AUC for repeated control response (1.2±0.1, n=14, data in Fig. 1C). Figure 1A illustrates representative traces of the extracellular single nerve fiber recording response of nodose C-fibers to esophageal distention with define pressure before and after superfusion with $GABA_B$ selective agonist baclofen in the concentration of 300 μM.

We next evaluated a structurally different and more potent $GABA_B$ agonist SKF97541 (Piqueras and Martinez 2004). We found that SKF97541 had no effect on mechanical response of nodose C-fibers. The AUC ratio for the mechanical response in the presence SKF97541 (3 μM, 30 min) was 1.2±0.1 (n=3) that was similar (p=0.9) to AUC ratio for repeated control response (1.2±0.1, n=14, data in Fig. 1C). These data indicate that the stimulation of $GABA_B$ receptors by baclofen or SKF97541 does not inhibit mechanical response in the esophageal nodose C-fibers.

Baclofen did not inhibit mechanical sensitization of nodose C-fibers induced by the stimulation of the adenosine A_{2A} receptors

Next we reasoned that although the stimulation of $GABA_B$ receptors does not inhibit mechanical response of nodose C-fibers, it may inhibit the sensitization of mechanical response in these C-fibers. We investigated the effects of $GABA_B$ agonists on mechanical sensitization induced *via* two distinct receptors for mediators relevant for esophageal pathophysiology: the adenosine A_{2A} receptor known to be coupled to activation of the cAMP/PKA pathway, and the histamine H_1 receptor known to be coupled to activation of the PKC pathway.

In control experiments the selective adenosine A_{2A} receptor agonist CGS21680 induced a reliable mechanical sensitization of nodose C-fibers (Fig. 2A). The AUC ratio for the mechanical response after pretreatment with CGS21680 (3 nM, 30 min) was 2.0±0.5 (n=6) that was higher ($p<0.05$) than AUC ratio for control repeated mechanical response 1.2±0.1 (n=14, data shown in Fig. 1C). Baclofen failed to inhibit CGS21680-induced sensitization (Fig. 2B-C). In the presence of baclofen (100 μM), incubation with CGS21680 (3 nM, 30 min) still resulted in sensitization of mechanical response. The AUC ratio for the mechanical response in this experiment was 2.3±0.6 (n=7) that was higher ($p<0.05$) than AUC ratio for control repeated mechanical response 1.2±0.1 (n=14, data shown in Fig. 1C). Thus baclofen did not inhibit CGS21680-induced sensitization of nodose C-fibers. In a limited set of experiments we also investigated the effect of SKF97541 (3 μM, 30 min) failed to inhibit sensitization of nodose C-fibers induced by CGS21680 (3 nM, 30 min). The AUC ratio for the mechanical response in this experiment was 2.1±0.6 (n=3) that was similar to sensitization evoked by CGS21680 (AUC ratio 2.0±0.5, n=6, data shown in Fig. 2A). This data indicate that the stimulation of $GABA_B$ receptors does not inhibit mechanical sensitization of nodose C-fibers induced *via* adenosine A_{2A} receptors.

Baclofen did not inhibit mechanical sensitization of nodose C-fibers induced by histamine

It has been previously shown that histamine evokes sensitization of nodose C-fibers that persists for at least 90 min (Yu *et al.* 2007). In the first set of experiments we investigated whether baclofen can reverse this sensitization. Nodose C-fibers were first sensitized by histamine and then the effect of baclofen on this sensitization was evaluated. As expected, histamine (100 μM, 30 min) induced a robust mechanical sensitization of nodose C-fibers (Fig. 3A). The AUC ratio

for the mechanical response after pretreatment with histamine was 2.9±0.7 (n=6) that was significantly higher ($p<0.01$) than AUC ratio for control repeated mechanical response (1.2±0.1, n=14, data shown in Fig. 1C). Baclofen (300 µM, 30 min) failed to reverse the mechanical sensitization established by histamine (Fig. 3A). The AUC ratio for the mechanical response in this experiment was 3.1±0.9 (n=6) that was significantly higher ($p<0.05$) than AUC ratio for control repeated mechanical response (1.2±0.1, n=14, data shown in Fig. 1C) and not different from the sensitization evoked by histamine (Fig. 3A).

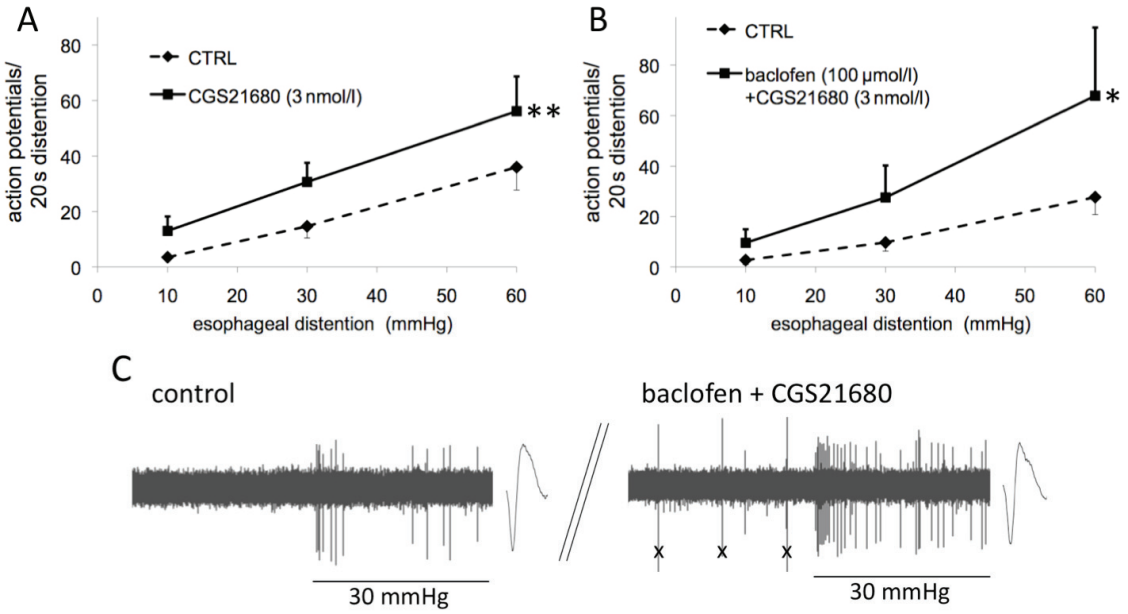

Fig. 2. Baclofen did not inhibit mechanical sensitization of nodose C-fibers induced by stimulation of the adenosine A_{2A} receptors. (**A**) Mechanical sensitization of nodose C-fibers evoked by the selective adenosine A_{2A} receptor agonist CGS21680 (n=6). (**B**) Baclofen did not inhibit mechanical sensitization by CGS21680 (n=7). * $p<0.05$, ** $p<0.01$, see text for details on statistical analysis. (**C**) Representative traces of the control response to distention and the sensitization of this response by CGS21680 (3 nM) in the presence of baclofen (100 µM).

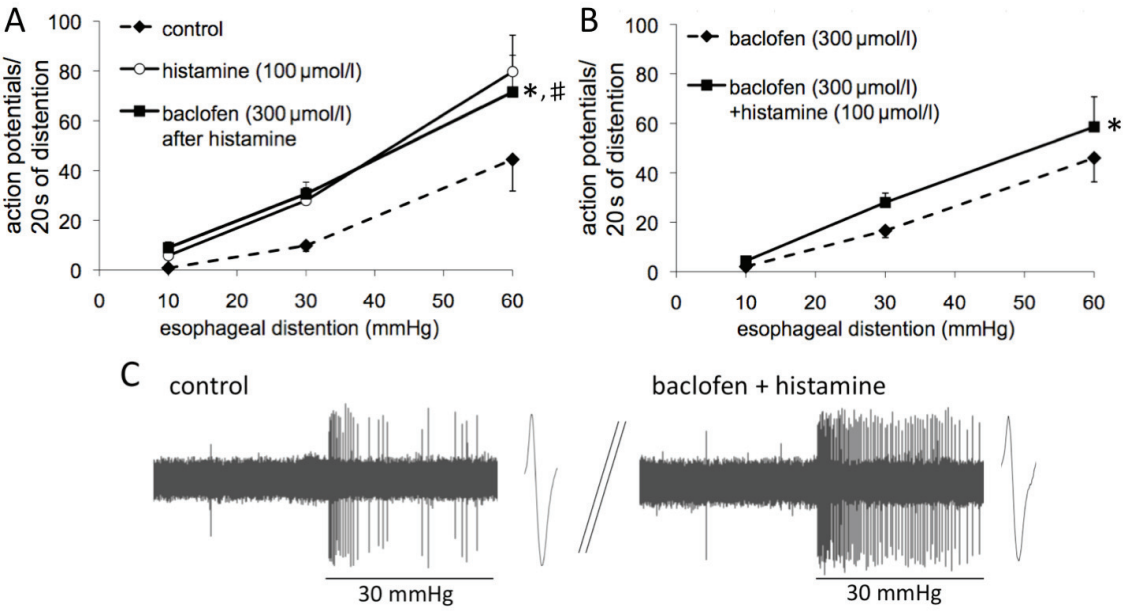

Fig. 3. Baclofen did not inhibit mechanical sensitization of nodose C-fibers induced by histamine. (**A**) Histamine induced mechanical sensitization of nodose C-fibers that was not reversed by baclofen (n=6, * $p<0.05$ histamine vs. control, # $p<0.05$ baclofen after histamine vs. control). (**B**) Pretreatment with baclofen prior to histamine was ineffective to prevent mechanical sensitization by histamine (n=7, * $p<0.05$). (**C**) Representative traces of the control response to distention and the sensitization of this response by histamine (100 µM) in the presence of baclofen (100 µM).

In the following study the nodose C-fibers were first pretreated with baclofen and then the effect of histamine in the presence of baclofen was evaluated. Pretreatment with baclofen (300 μM, 30 min) failed to prevent mechanical sensitization induced by histamine (100 μM, 30 min) (Fig. 3B). The AUC ratio for the mechanical response in this experiment was 1.5±0.2 (n=7) that was significantly higher ($p<0.05$) than AUC ratio for control repeated mechanical response (1.2±0.1, n=14, data shown in Fig. 1C). Although numerically the magnitude of sensitization is lower, it is not significantly different (p=0.1) from the sensitization evoked by histamine (Fig. 3A). This data indicate that baclofen neither reversed, nor prevented histamine-induced sensitization of nodose C-fibers.

Baclofen did not change the pattern of mechanically-evoked action potential discharge

We also investigated whether the stimulation of $GABA_B$ receptors changes the time course pattern of distention-evoked action potential discharge that would change the information transmitted by nodose C-fibers to central nervous system. As described in methods we calculated the dynamic response index (see methods details) for the distention with 30 mmHg that was found most sensitive to changes in mechanical responsiveness (Yu *et al.* 2007, Yu and Ouyang 2009). We observed that the dynamic response index is reproducible in control conditions (i.e. two repeated distentions, 0.63±0.05 vs. 0.58±0.05, n=20, p=0.5, analysis of the data in Fig. 1C). We found that baclofen did not change the pattern of action potential discharge in the baseline or sensitized conditions. Figure 1B illustrates that the time course of the action potential discharge evoked by distention before and after superfusion with baclofen (300 μM) was not affected. The dynamic response index was 0.62±0.12 in the presence of baclofen (100 μM) (data in Fig. 1D, n=10, p=0.9 compared to repeated controls) and 0.55±0.09 in the presence of baclofen (300 μM) (data in Fig. 1B and 1E, n=13, p=0.4 compared to repeated controls).

In the experiments with the adenosine A_{2A} agonist, the dynamic response index was 0.56±0.09 (n=6) in the presence of CGS21680 (3 nM) and 0.54±0.10 (n=7) in the presence of CGS21680 (3 nM) and baclofen (100 μM) (p=0.8, analysis of the data in Fig. 2.) We also found that baclofen did not change the pattern of action potential discharge in histamine-induced sensitization (evaluated by dividing the peak frequency by the total number of action potentials in 20 s of the distention for technical reasons indicated in methods). This parameter was 0.26±0.6 in the presence of histamine (100 μM) and 0.25±0.5 in the presence of histamine (100 μM) and baclofen (300 μM) (p=0.9, n=6, analysis of the data in Fig. 3A).

Discussion

We found that $GABA_B$ agonists did not inhibit mechanical activation of vagal esophageal nodose C-fibers. We also found that $GABA_B$ agonists did not inhibit mechanical sensitization of nodose C-fibers induced by stimulation of the adenosine A_{2A} and histamine H_1 receptors that couple to different G proteins, G_s and G_q, respectively. Our data indicate that the effect of $GABA_B$ agonists on esophageal reflexes described previously *in vivo* are probably not mediated by the action on peripheral nerve terminals of nodose C-fibers in the esophagus.

The esophageal vagal nociceptive fibers have been only recently described in detail (Yu *et al.* 2005). Because of that, the reflexes and perceptions mediated by vagal esophageal nociceptors have not been elucidated yet. Nonetheless, vagal nociceptive C-fibers have been extensively studied in the neighbouring airways and lungs, and are well recognized to trigger and modulate many defensive respiratory reflexes as well as respiratory sensations (Canning and Chou 2009). It is therefore reasonable to predict that the esophageal vagal nociceptors contribute to initiation and regulation of esophageal motor reflexes and to sensations from the esophagus. We therefore reasoned that inhibition of vagal nodose C-fibers by $GABA_B$ agonists could contribute to benefitial effects of $GABA_B$ agonists observed in some patients. For example, the $GABA_B$ agonist baclofen has been widely reported to reduce the frequency of reflux and improve symptoms in patients with gastroesophageal reflux disease (Blackshaw *et al.* 1999, Boeckxstaens 2011, Koek *et al*. 2003, Lehmann 2009, Vela *et al.* 2003, Zhang *et al*. 2002).

We initially evaluated the effect of baclofen on mechanical response of nodose C-fibers. Because we did not observe any inhibitory effect, we hypothesized that activation of $GABA_B$ may be effective to inhibit mechanical sensitization of esophageal nociceptors. We elected to evaluate the effect of baclofen on mechanical sensitization evoked *via* activation of the adenosine A_{2A} receptors and histamine H_1 receptors for two reasons.

Firstly, adenosine and histamine are significant pronociceptive and proinflammatory mediators in the esophagus. Several clinical studies demonstrated that adenosine is important for pathogenesis of esophageal non-cardiac chest pain (Chahal and Rao 2005, Achem 2007, Remes-Troche *et al*. 2009). Recent research also indicates that the accumulation and activation of mast cells (accompanied by the release of histamine) can be induced by inflammation, reflux acid, and is also found in another esophageal disorder eosinophilic esophagitis (Nielsen *et al.* 2006, Lucendo *et al.* 2009, Vicario *et al.* 2010). Indeed, we have reported that activation of the adenosine A_{2A} receptors and the histamine H_1 receptors induces sensitization of esophageal nociceptors (Yu *et al.* 2007, Ru *et al.* 2011). Secondly, the adenosine A_{2A} and histamine H_1 receptors are G-protein coupled receptors (GPCRs) that couple to two different intracellular pathways. The most commonly recognized signal transduction mechanism for A_{2A} receptors is the activation of adenylate cyclase that implies coupling with the G_s protein and activation of a cAMP-dependent protein kinase (Ralevic and Burnstock 1998), while the primary mechanism by which histamine H_1 receptors produce functional responses in cells is the activation of phospholipase C *via* a pertussis toxin-insensitive G-protein that is probably related to the $G_{q/11}$ (Hill *et al.* 1997).

We have reported that activation of the selective adenosine A_{2A} receptor agonist CGS21680 induces sensitization of esophageal C-fibers (Ru *et al.* 2011). The sensitizing effect of CGS21680 (0.003 μM) was completely abolished by the selective A_{2A} antagonist SCH58261 (0.1 μM) (data not shown) indicating that this effect is mediated by A_{2A} receptor. In the present study we found that baclofen (100 μM) neither reversed, nor prevented mechanical sensitization by CGS21680 in nodose C-fibers. We thus conclude that the stimulation of $GABA_B$ receptors does not inhibit mechanical sensitization of nodose C-fibers induced *via* the adenosine A_{2A} receptors.

In histamine experiments we used the lowest effective concentration of histamine (100 μM) based on our previous study (Yu *et al.* 2007). In the referenced study the effect of histamine (100 μM) was abolished by the selective H_1 receptor antagonist pyrilamine (1 μM) demonstrating that this effect is mediated by the histamine H_1 receptor. In the present study we found that baclofen even in the concentration of 300 μM failed to prevent the histamine-induced sensitization and also failed to reverse sensitization established by pretreatment with histamine (100 μM). We thus conclude that the stimulation of $GABA_B$ receptors does not inhibit mechanical sensitization of nodose C-fibers induced *via* the histamine H_1 receptors. Combined these data indicate that the stimulation of $GABA_B$ receptors does not inhibit mechanical sensitization due to activation of sensitizing pathways initiated by G_s and G_q receptors in esophageal nodose C-fibers.

In addition to baclofen, we also evaluated another $GABA_B$ selective agonist SKF97541 (Piqueras and Martinez 2004). Consistent with the baclofen studies we found that SKF97541 had no effect on mechanical response of nodose C-fibers and failed to inhibit the adenosine A_{2A}-mediated sensitization of nodose C-fibers. Thus, our conclusion that the $GABA_B$ selective agonists do not inhibit mechanical response of nodose C-fibers is based on the use of two different $GABA_B$ receptor selective agonists.

In the present study we evaluated the question whether $GABA_B$ agonists inhibit the mechanically-induced (mechanical) activation of the nodose C-fibers in the esophagus. We addressed this question by using our validated single unit recordings of nerve activity originating from the C-fiber terminals in the esophagus in an isolated *ex vivo* esophagus-nerve preparation. This techniques offers a number of advantages including: 1) the evaluation of the activity originating from the relevant mechanotransduction site, the nerve terminal in the tissue, 2) single fiber (unit) activity is recorded (very good signal to noise ratio), 3) *ex vivo* preparation allowing for tight control of the stimuli (i.e. reproducible esophageal distention with desired pressure and duration without confounding secondary motor reflex changes evoked by distention *in vivo*), 4) tight control of the drug concentration (equilibrium system with the drug access to the nerve terminals confirmed by response to other agonists), 5) extensive information available on this preparation (Yu *et al.* 2005, Yu *et al*. 2007, Yu and Ouyang 2009, Ru *et al.* 2011) and specifically on the neurobiology of esophageal nodose C-fibers (reviewed in Kollarik *et al.* 2010). Thus, this technique is optimal for the study of pharmacological questions such as those investigated in our study.

The lack of certain local (such as blood dependent) and reflex (such as reflex contraction) secondary effects may be disadvantageous depending on the question addressed, such as what is the response to a given stimulus *in vivo*. For example, if one speculates that

$GABA_B$ agonists can inhibit C-fibers by acting on some other cell type(s) to release an inhibitory signal for C-fibers, such pathway may not be suitably preserved *ex vivo*, especially if some blood components are required. However, we are not aware of a $GABA_B$-receptor mediated inhibition that would require involvement of an additional cell type. Also, with this technique, the measurement of membrane potential of the nerve terminal is not available, so the changes in membrane properties often useful for mechanistic studies cannot be evaluated. Instead, an integrated response in the form of action potentials (that *in vivo* constitutes the input to CNS) is recorded.

Nonetheless, while we are confident in the conclusion that the $GABA_B$ agonists do not directly inhibit esophageal nodose C-fibers under the conditions tested, the possibility that the signalling through these nerves is reduced by $GABA_B$ agonists in patients with GERD cannot be ruled out. For example, speculatively, $GABA_B$ activation may inhibit some events that (directly or through reflexes) trigger or enhance activation of nodose C-fibers in patients with GERD. Alternatively, C-fibers in these patients may undergo plastic changes that render them sensitive to $GABA_B$ agonists. Unfortunately, the specific information on the C-fiber biology in patients with GERD that would allow addressing these speculations is not available yet.

The lack of effect of $GABA_B$ agonists on esophageal nodose nociceptors is unlikely to be explained by diffusion barriers that would prevent baclofen from reaching the $GABA_B$ receptors on the nociceptive nerve terminals in the esophagus. We demonstrated that the drugs delivered in an identical manner as baclofen could easily modulate nodose C-fibers. Specifically, we found that nodose C-fibers in the esophageal preparation readily responded to capsaicin (1 μM), or were readily sensitized by A_{2A} and H_1 receptors agonists, confirming the drug accessibility to the nerve terminals. The $GABA_B$ agonists were dosed well above their reported EC_{50s}, baclofen (100, 300 μM) or SKF9754 (3 μM) so that the lack of effect of these drug cannot be attributed to an insufficient concentrations. Indeed, these concentrations of baclofen were found effective to inhibit non-nociceptive low threshold (tension) mechanosensors in previous studies (Blackshaw *et al.* 1999, Page and Blackshaw 1999, Smid and Blackshaw 2000, Smid *et al.* 2001, Zagorodnyuk *et al.* 2002).

In conclusion, our data show that the activation of $GABA_B$ receptors does not inhibit mechanical activation of esophageal nodose C-fibers in baseline state and certain sensitized states, and indicates that mechanical inhibition of nodose C-fibers in the esophagus does not contribute to beneficial effects of $GABA_B$ agonists in patients with esophageal diseases.

Conflict of Interest

There is no conflict of interest.

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