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Chapter XX

GAL-021 and GAL-160 are efficacious in rat models of obstructive and central sleep apnea and inhibit BK_{Ca} in isolated rat carotid body glomus cells

Mark L. Dallas, Chris Peers, Francis J. Golder, Santhosh Baby, Ryan Gruber and D. Euan MacIntyre

Abstract. GAL-021 and GAL-160 are alkylamino triazine analogues, which stimulate ventilation in rodents, non-human primates and (for GAL-021) in humans. To probe the site and mechanism of action of GAL-021 and GAL-160 we utilized spirometry in urethane anesthetized rats subjected to acute bilateral carotid sinus nerve transection (CSNTX) or sham surgery. In addition, using patch clamp electrophysiology we evaluated ionic currents in carotid body glomus cells isolated from neonatal rats. Acute CSNTX markedly attenuated and in some instances abolished the ventilatory stimulant effects of GAL-021 and GAL-160 (0.3 mg/kg IV), suggesting the carotid body is a/the major locus of action. Electrophysiology studies, in isolated Type I cells, established that GAL-021 (30 μ M) and GAL-160 (30 μ M) inhibited the BK_{Ca} current without affecting the delayed rectifier K⁺, leak K⁺ or inward Ca²⁺ currents. At a higher concentration of GAL-160 (100 μ M), inhibition of the delayed rectifier K⁺ current and leak K⁺ current were observed. These data are consistent with the concept that GAL-021 and GAL-160 influence breathing control by acting as peripheral chemoreceptor modulators predominantly by inhibiting BK_{Ca} mediated currents in glomus cells of the carotid body.

Keywords: GAL-021, GAL-160, BK_{Ca} channel, carotid body, carotid sinus nerve, peripheral chemoreceptor modulators

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XX.2 Introduction

Predicated on the concept that agents which modulate the drive to breathe represent a rational approach towards the development of therapeutics for breathing control disorders such as drug-induced respiratory depression and sleep apnea, a phenotypic screening approach was used to identify ventilatory stimulant compounds. Starting from precedented agents, such as doxapram and almitrine, that are effective ventilatory stimulants in humans (Golder et al 2013), a diversity of molecular scaffolds were designed, constructed, evaluated and further optimized to incorporate drug-like properties relating to potency, efficacy, specificity and tolerability. These efforts culminated in the identification of 2 development candidate compounds, the alkylamino triazine derivatives GAL-021 and GAL-160.

Upon intravenous administration by bolus or infusion to rats (Baby et al, 2012a) or cynomolgus monkeys (Golder et al, 2012) GAL-021 elicits dose-dependent increases in minute ventilation and shows robust, dose-dependent, reversal of opioid (morphine/fentanyl/alfentanil), benzodiazepine (midazolam), and anaesthetic (isoflurane/propofol)-induced respiratory depression (Baby et al, 2012a, Golder et al, 2013). GAL-021 recently has been shown to stimulate ventilation and to reverse opioid-induced respiratory depression in human volunteers (McLeod et al, 2014, Roozekrans et al, 2014). Unlike opioid receptor antagonists, GAL-021 does not reverse or compromise opioid analgesia in rats or humans (Baby et al, 2012a; Roozekrans et al, 2014). GAL-160 displays a similar profile of activity to GAL-021 with respect to spirometry and opioid-induced respiratory depression in rodents, but has higher oral bioavailability in preclinical species. In rodent models of central and obstructive sleep apnea, both GAL-021 and GAL-160 demonstrate beneficial effects on apnea frequency and duration, and reduce the desaturation sequelae without compromising sleep architecture (Gruber et al, 2013; Hewitt et al, 2014).

We have shown previously that GAL-021-induced ventilatory stimulation in rats is associated with increased carotid sinus nerve and phrenic motoneuron activity (Baby et al 2012b), suggesting that these alkylamino triazine derivatives may act, at least in part, at the carotid body to promote afferent signaling to the brainstem respiratory centers and resultant efferent signaling to the upper airways and diaphragm, consistent with increased respiratory drive. Key to this signaling cascade are the complement of hypoxic sensing ion channels expressed in the carotid body. The molecular identity of the carotid body K^+ channel(s) which is/are inhibited by hypoxia remains a matter of debate. Controversies and interpretation conflicts arise inter alia because of species and age differences in the isolated type I cells evaluated, type of preparation used for investigation (e.g. single cell vs cluster), and configuration used for single channel recording (Buckler & Honore, 2004; Peers et al, 2010; Kim, 2013). In the rat carotid body much evidence points to the presence and functional importance of BK_{Ca} and TASK channels in chemotransduction (Buckler, 2007; Peers et al, 2010; Kim et al, 2012). There are few reports relating to human carotid body chemotransduction, although a recent report documents that the human carotid body transcriptome contains BK_{Ca} and TASK-1 channels (Mkrtchian et al, 2012). To probe further the site and mechanism of action of GAL-021 and GAL-160, we compared the evoked ventilatory stimulation in rats subjected to acute bilateral carotid sinus nerve transection or sham surgery. In addition we evaluated the effects of GAL-021 and GAL-160 on K^+ and Ca^{2+} currents using whole cell patch clamp electrophysiology in isolated rat carotid body type I glomus cells.

XX.3 Methods

XX.3.1 Spirometry studies

Male adult Sprague Dawley rats were used for these studies. Animals were initially anesthetized with 2% isoflurane. The femoral artery and vein were cannulated to permit monitoring blood pressure and drug administration. The cervical trachea was cannulated and connected to a pneumotachometer (MLT1L, AD Instruments, CO) to measure respiratory flow. The respiratory flow waveform was used to measure respiratory rate (RR) from cycle period, and integrated to calculate tidal volume (V_T). Minute volume (\dot{V}_E) was calculated as the product of RR and V_T . After these procedures, isoflurane was discontinued and replaced by urethane anesthesia (1.8 g/kg IV). Stable baseline values of cardiorespiratory parameters and arterial blood gases were confirmed before commencing the experimental protocol. Minute ventilation was measured before and after vehicle (saline), GAL-021 (0.3 mg/kg IV) and GAL-160 (0.3 mg/kg IV) injection. GAL-021 and GAL-160 were administered by slow IV bolus to separate groups of animals. Next, the carotid sinus nerves were transected bilaterally at the point where they branch off from the glossopharyngeal nerve and the protocol repeated. Sham-operated animals were included as controls. The ventilatory response to hypoxia ($FiO_2 = 0.10$, 3 min duration) was used to confirm functional denervation of the carotid bodies.

XX.3.2 Glomus (Type I) Cell isolation

To obtain carotid body glomus cells, neonatal rats (10 – 14 days) were killed and carotid bodies rapidly removed and placed in ice cold, phosphate buffered saline without Ca^{2+} or Mg^{2+} (Invitrogen). These were then enzymatically dissociated and cultured as described previously (Peers, 1990; Wyatt and Peers, 1993)

XX.3.2 Electrophysiology

Fragments of coverslip with attached cells were transferred to a continuously perfused (3-5 ml/min) recording chamber mounted on the stage of an Olympus CK40 inverted microscope. All experiments were carried out at room temperature ($22 \pm 1^\circ C$). Whole cell patch clamp recordings were then obtained from type I cells in voltage clamp mode. Patch pipettes had resistances of 4-6M Ω . Series resistance was monitored after breaking into the whole cell configuration throughout the duration of experiments. If a significant increase occurred (>20%), the experiment was terminated. Signals were acquired using a Axopatch 200B (Axon Instruments, Inc., Foster City, CA) controlled by Clampex 9.0 software via a Digidata 1322A interface (Axon Instruments, Inc., Foster City, CA). Data were filtered at 1 kHz and digitized at 5 kHz.

K⁺ currents

To determine the effects of the test compounds on the K⁺ currents we employed several protocols and solutions. For all experiments unless stated the standard perfusate (pH 7.4) was composed of (in mM): NaCl (135); KCl (5); MgCl₂ (1.2); Hepes (5); CaCl₂ (2.5); D-glucose (10). The intracellular solution (pH 7.2) consisted of in (mM): KCl (117); NaCl (10); EGTA (11); MgCl₂ (2); CaCl₂ (1); HEPES (11); NaATP (2). All cells were voltage clamped at -70mV and K⁺ currents were evoked with a series of depolarising steps from -80mV to

+60mV (100ms, 10mV increments). In order to examine the effects on the BK_{Ca} current, a ramp protocol -100 mV to +60 mV (1s) was also employed, followed by a 250ms step depolarisation to +60mV. In addition a similar perfusate with the exception of 6mM MgCl₂ and 0.1mM CaCl₂ was used to inhibit the BK_{Ca} component of the whole cell K⁺ current, as described previously (Peers 1990; Peers & Green, 1993). To investigate the 'leak' K⁺ current, a ramp protocol (-100mV to -40mV) was employed as described previously (Wyatt et al, 2007). 10mM BaCl₂ was used to block of the leak K⁺ current component.

Ca²⁺ currents

For Ca²⁺ channel experiments the standard perfusate (pH 7.4) was composed of (in mM): NaCl (110); CsCl (5); MgCl₂ (0.6); Hepes (5); BaCl₂ (10); D-glucose (10); TEA (20mM). The intracellular solution (pH 7.2) consisted of in (mM): CsCl (130); NaCl (10); EGTA (11); MgCl₂ (2); CaCl₂ (0.1); HEPES (10); MgATP (2). To determine the effects of the test compounds on the Ca²⁺ currents cells were clamped at -80mV and Ca²⁺ currents evoked with a series of depolarising steps from -80mV to +40mV.

Chemicals

All chemicals were from Sigma-Aldrich (Poole, UK) unless stated otherwise. All test compounds were provided by Galleon Pharmaceuticals and made up as stock solutions on the day of the experiment and diluted (DMSO) to the stated concentration in the perfusate.

Data analysis

Offline analysis was carried out using the data analysis package Clampfit 9 (Axon Instruments) and data are expressed as mean ± SEM. P values are from 2-tailed Student's t-test, where P<0.05 was considered significant.

XX.4 Results

XX4.1 Role of the carotid body/carotid sinus nerve in the ventilatory stimulant effects of GAL-021 and GAL-160

In rats subjected to sham surgery ventilatory stimulation was evident following hypoxia (FiO₂ = 0.1, 3 min duration), and following administration of GAL-021 (0.3 mg/kg IV) or GAL-160 (0.3 mg/kg IV). When compared to responses evoked in the sham surgery animals, the ventilatory stimulant effects of hypoxia and of GAL-021 and GAL-160 were markedly inhibited (GAL-021) or virtually abolished (GAL-160, hypoxia) in rats subjected to acute carotid sinus nerve transection. Figure XX.1.

Abrogation of the \dot{V}_E increase elicited by hypoxia is confirmation that the acute carotid sinus nerve transection procedure was successful. Thus, attenuation of responses evoked by GAL-021 and GAL-160 in carotid sinus nerve transected animals suggests that afferent signaling from the carotid body to the brainstem is prerequisite for a major component of the ventilatory stimulation elicited by these agents.

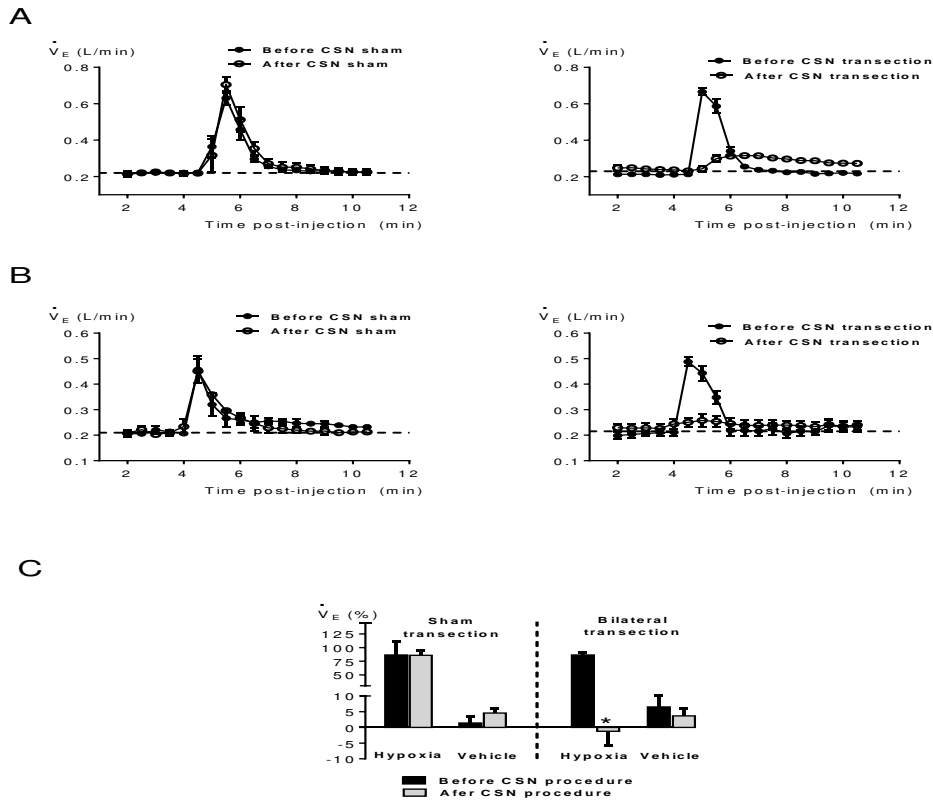


Fig. XX.1: The effects of GAL-021, GAL-160, vehicle (saline) and hypoxia on minute ventilation in anesthetized rats before and after sham transection or bilateral transection of the carotid sinus nerve (CSN).

The ventilatory stimulant effects of GAL-021 (0.3 mg/kg IV) (A), GAL-160 (0.3 mg/kg IV) (B) and Hypoxia (FiO₂ = 0.10, 3 min duration) (C) were markedly reduced after denervation of the carotid bodies. * significantly different to the intact state.

XX4.2 GAL-021 and GAL-160 inhibit K⁺ currents in glomus cells

Whole cell K⁺ currents were recorded from glomus cells before and during exposure to GAL-021 (30 μM) and GAL-160 (30 μM). As shown in Figure XX.2 GAL-021 (30 μM) and GAL-160 (30 μM) significantly inhibited outward K⁺ currents in glomus cells. GAL-021 caused a 62.3 ± 8.8% inhibition at +60mV (n=8, P<0.001 compared to vehicle control), in a similar fashion GAL-160 elicited 58.6 ± 11.3% inhibition at +60mV (n=12, P<0.005 compared to vehicle control). Under standard recording conditions, these K⁺ currents are composed of BK_{Ca} channels and a delayed rectifier component, and the magnitude of the BK_{Ca} component is determined, at least in part, by influx of Ca²⁺ via voltage gated Ca²⁺ channels (Peers, 1990).

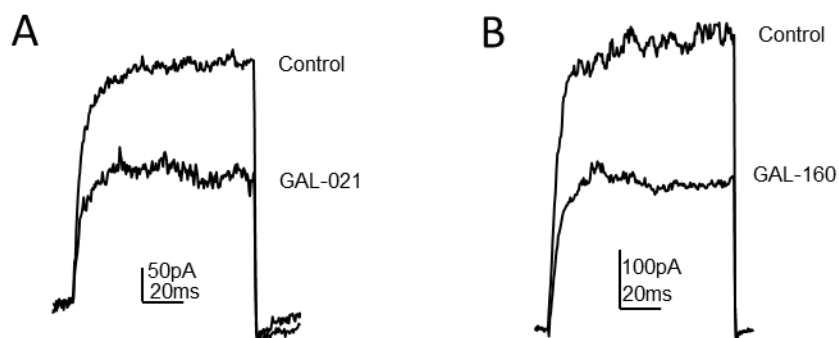


Figure XX.2: GAL-021 and GAL-160 inhibit whole cell K⁺ currents in glomus cells.

(A) Example whole-cell K⁺ currents evoked by a step depolarization (-80mV to +60mV, 100ms) before (control) and during exposure to 30μM GAL-021. (B) Example whole-cell K⁺ currents evoked by a step depolarization (-80mV to +60mV, 100ms) before (control) and during exposure to 30μM GAL-160.

XX4.3 GAL-021 and GAL-160 inhibit BK_{Ca} currents in glomus cells.

To evaluate potential effects of GAL-021 and GAL-160 on the delayed rectifier component independent of the BK_{Ca} component, studies were conducted in a high Mg²⁺/low Ca²⁺ milieu to inactivate BK_{Ca}. As shown in Figure XX3, during BK_{Ca} inhibition, GAL-021 (30μM) and GAL-160 (30μM) do not significantly impair outward K⁺ current, suggesting that at these concentrations their major effects are on BK_{Ca}. When tested at a higher concentration, modest inhibition of outward K⁺ current by GAL-160 (100μM) was evident (data not shown).

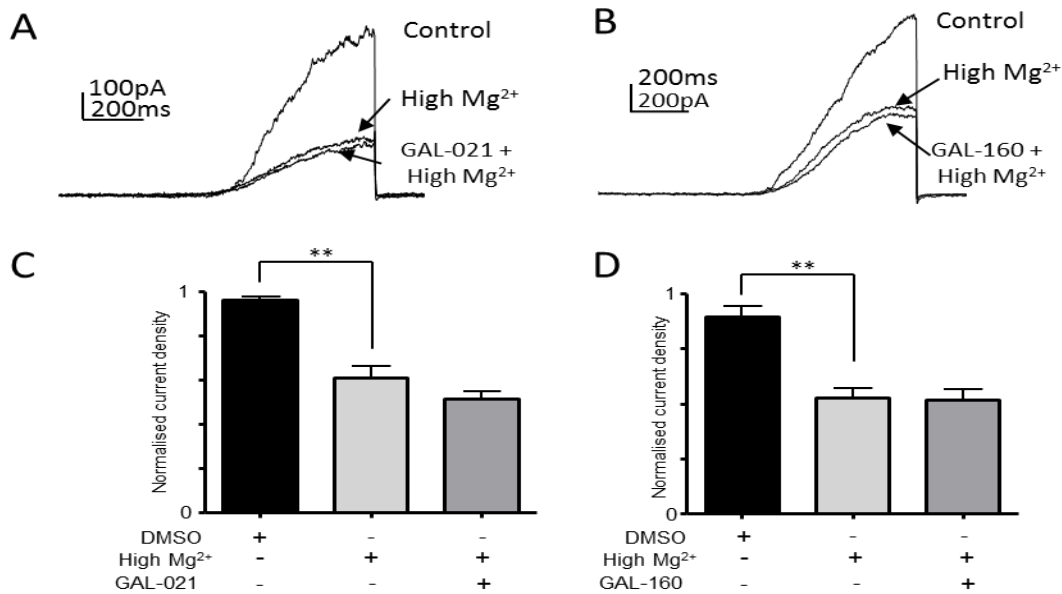


Figure XX.3. GAL-021 and GAL-160 inhibit BK_{Ca} currents in glomus cells. (A) Example traces showing ramp depolarizations (-100mV to +60mV, 1s) in control, in the presence of high Mg²⁺ / low Ca²⁺ solution (High Mg²⁺) and in presence of GAL-021 and (B) another example utilizing GAL-160. In both examples cells were exposed initially to the high Mg²⁺ / low Ca²⁺ solution, and then GAL-021 and GAL-160 in the continued presence of the high Mg²⁺ / low Ca²⁺ solution. (C, D) Bar graph showing mean normalized current densities

(determined at +60mV from 4-6 cells in each case) and the relative effects of DMSO, high Mg^{2+} / low Ca^{2+} solution alone, and GAL-021 and GAL-160 in the presence of high Mg^{2+} / low Ca^{2+} solution as indicated. ** indicates $P < 0.01$.

XX4.4 GAL-021 and GAL-160 do not inhibit Ca^{2+} currents in type I cells

As, under the recording conditions utilized, BK_{Ca} activity is dependent upon Ca^{2+} influx (Peers 1990), it remained possible that inhibition of BK_{Ca} by GAL-021 and GAL-160 could arise secondarily to effects on Ca^{2+} influx. When effects on whole-cell Ca^{2+} currents were measured in type I cells, neither GAL-021 (30 μ M) nor GAL-160 (30 μ M) altered Ca^{2+} current. GAL-021 led to -5.6 ± 4.8 % change from control (n=3, $P > 0.01$, at 0mV). GAL-160 led to a -3.9 ± 6.1 % change from control (n=5, $P > 0.01$, at 0mV)

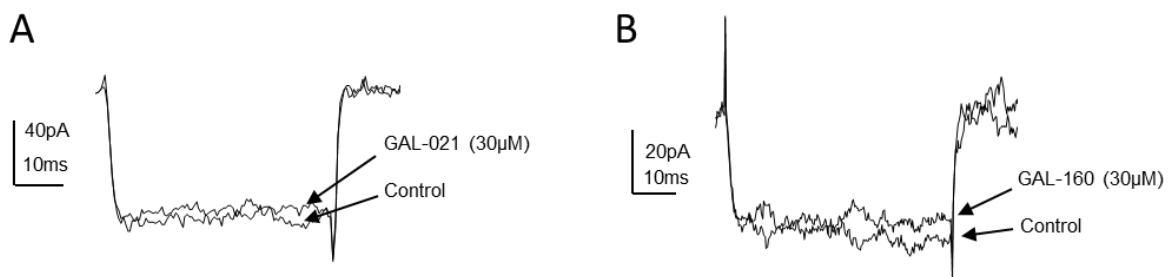


Figure XX.4. GAL-021 and GAL-160 do not alter Ca^{2+} currents in type I cells. (A, B) Example Ca^{2+} currents recorded before and during exposure to GAL-021 (30 μ M) and GAL-160 (30 μ M).

XX4.5 GAL-021 and GAL-160 do not inhibit leak K^{+} currents in glomus cells.

Leak K^{+} currents, carried by TASK-channels are implicated in carotid body activation by physiological stimuli, such as hypoxia (Buckler & Turner, 2013, Kim et al 2012), as well as by doxapram (Cotten et al, 2006). Moreover, small molecule TASK inhibitors are ventilatory stimulant in rats (Cotten, 2013). Accordingly, we evaluated the effects of GAL-021 and GAL-160 on the Ba^{2+} sensitive leak K^{+} current in glomus cells. In comparison to vehicle (DMSO) treatment, neither GAL-021 (30 μ M) nor GAL-160 (30 μ M) exerted significant effects on the Ba^{2+} sensitive leak K^{+} current. In the presence of GAL-160 (100 μ M), this current was significantly reduced.

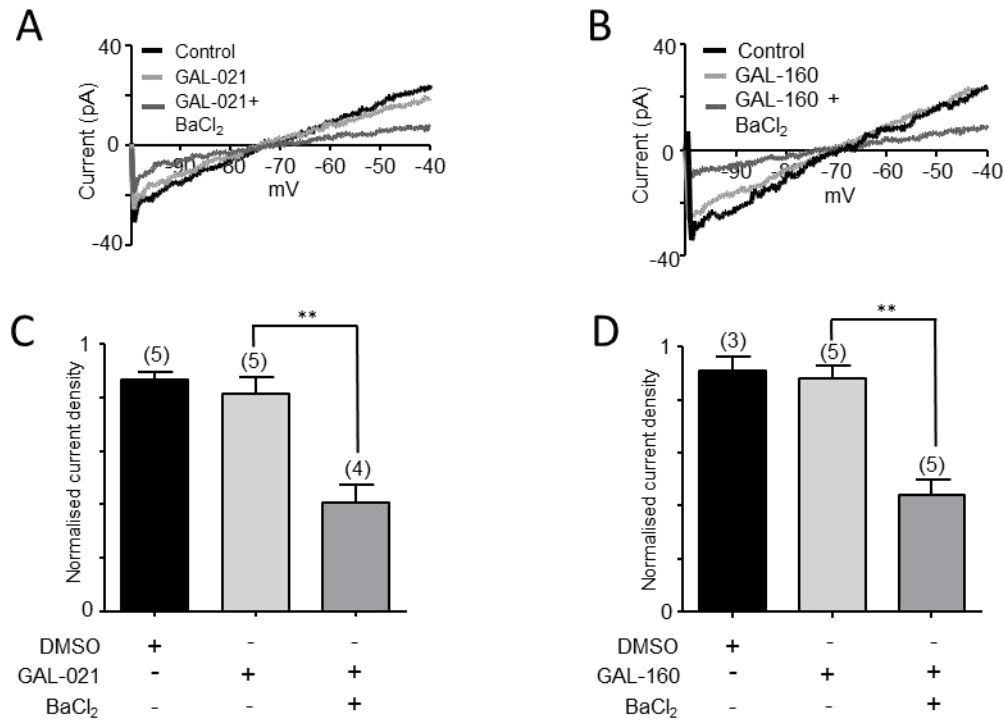


Figure XX.4. GAL-021 and GAL-160 do not inhibit leak K^+ currents in glomus cells. (A, B) Example leak currents evoked by ramp depolarizations around the resting membrane potential (-100mV to +40mV) under control conditions, in the presence of (A) GAL-021 (30 μ M) and (B) GAL-160 (30 μ M) and in the subsequent presence of BaCl₂ (10mM). (C) Bar graph showing mean normalized current densities and the relative effects of vehicle (DMSO), GAL-021 (30 μ M) and BaCl₂ (10mM) in the presence of GAL-021 (30 μ M). (D) Bar graph showing mean normalized current densities and the relative effects of vehicle (DMSO), GAL-160 (30 μ M) and BaCl₂ (10mM) in the presence of GAL-160 (30 μ M). Numbers of cells recorded are indicated above each bar. ** indicates $P < 0.01$.

XX.5 Discussion

Marked attenuation (as with GAL-021) or abolition (as with GAL-160) of the ventilatory stimulant effects of these alkylamino triazine analogues following acute bilateral carotid sinus nerve transection in rats, allied to increases in carotid sinus nerve activity during GAL-021 administration in rats (Baby et al, 2012b) indicate that the carotid body is a/the major peripheral site of action of these agents.

Although the molecular mechanisms underlying carotid body activation are not fully understood, they are generally accepted to converge on exocytotic release of neurotransmitters (e.g. acetylcholine, dopamine, ATP, neuropeptides) some of which activate the carotid sinus nerve (Nurse 2010). Exocytosis is triggered by an elevation in the intracellular free Ca^{2+} concentration. This derives predominantly via influx of extracellular Ca^{2+} through L-type Ca^{2+} channels, which are activated by membrane depolarization. Such depolarization is believed to arise from inhibition of certain K^+ channels, the expression of which varies across species (Weir et al, 2005; Peers et al, 2010.). There is consensus that

BK_{Ca} and TASK/leak channels are expressed in rodent and human carotid bodies, although these have only been characterized electrophysiologically in glomus cells of rodents (Buckler 2007; Peers et al, 2010; Turner and Buckler, 2013).

GAL-021 and GAL-160 exerted inhibitory effects on ionic currents in rat carotid body glomus cells. At 30 μ M both compounds inhibited BK_{Ca} currents without affecting the delayed rectifier K⁺ current or leak K⁺ current. At higher concentrations GAL-160 (100 μ M) showed some inhibition of the delayed rectifier K⁺ current and the leak K⁺ current. GAL-021 and GAL-160 had no effect on the inward Ca²⁺ current. Hence, the effects of both compounds on BK_{Ca} cannot be secondary to an inhibition of Ca²⁺ influx. Thus, these data suggest that GAL-021 and GAL-160 influence breathing control in rats by acting as peripheral chemoreceptor modulators predominantly by inhibiting BK_{Ca} channels in glomus cells of the carotid body. In this regard, GAL-021 and GAL-160 share some similarity with almitrine and doxapram. Almitrine inhibits rat glomus cell BK_{Ca} currents (IC₅₀ \sim 200nM) without altering voltage dependent K⁺, Na⁺, or Ca²⁺ currents (Peers and O'Donnell, 1990; Lopez-Lopez et al, 1998). To our knowledge, the effects of almitrine on leak K⁺ currents or TASK channels have not been tested. BK_{Ca} current in rat glomus cells is also inhibited by doxapram (IC₅₀ \sim 5 μ M) (Peers, 1991), which also inhibits current through cloned rat TASK channels expressed in oocytes with IC₅₀ \sim 400nM for TASK-1 and IC₅₀ \sim 47 μ M for TASK-3 (Cotten et al., 2006).

One area of controversy regarding glomus cell BK_{Ca} involvement in chemotransduction elicited by hypoxia relates to uncertainty as to whether the BK_{Ca} channel contributes to glomus cell resting membrane potential under physiological conditions and whether channels are open and operative, and hence subject to modulation/inhibition, at the negative resting membrane potentials (Buckler, 2007; Peers and Wyatt, 2007). One might question whether this concern also applies to carotid body activation and afferent nerve signaling elicited by exogenous small molecule BK_{Ca} inhibitors. Recently, there has been described a family of leucine-rich repeat containing proteins that act as auxiliary or accessory subunits, termed γ subunits, of the BK_{Ca} channel. These γ subunits regulate the gating properties of BK_{Ca} channels leading to channel opening even at negative resting membrane potentials and, depending upon the subunit expressed, confer tissue or cell specificity in channel modulation (Yan and Aldrich, 2012). Moreover, γ subunits reportedly also impact the pharmacology of channel opening, but whether they modify channel inhibition remains to be determined. The presence of γ subunits or their profile of expression in carotid body glomus cells remains to be determined. This adds an additional layer of complexity to our quest towards understanding the pharmacology and biophysics of BK_{Ca} channel modulation which are already known to be influenced by the subunit composition of the channel, by splice variants in the α subunit, by the nature of the β auxiliary subunit, where β 1 – β 4 subunits are expressed in a tissue-specific manner, and by the basal phosphorylation status (Widmer et al 2003; Ross et al, 2011; Hoshi et al, 2013).

In aggregate, the data suggest that BK_{Ca} inhibition is a major component of the mechanism of action of GAL-021 and GAL-160, but do not preclude that additional mechanism(s) may also be operative. Whether GAL-160 affects other processes (e.g. mitochondrial bioenergetics, AMPK activation) implicated in chemoreception (Ross et al, 2011; Buckler and Turner, 2013) remains to be determined. In addition, whether these actions of GAL-021 and GAL-160 on carotid body BK_{Ca} channels underlie all of their effects on indices of breathing control will require more detailed correlative pharmacokinetic-pharmacodynamic studies in which biological effects at free drug concentrations in the relevant biological compartment are

related to concentrations which affect electrophysiological parameters in the putatively relevant native ion channels.

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