Paracrine Signaling in Glial-Like Type II Cells of the Rat Carotid Body

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Abstract

The carotid body (CB) chemosensory complex uses ATP as a key excitatory neurotransmitter that is the main contributor to the sensory discharge during acute hypoxia. The complex includes receptor type I cells, which depolarize and release various neurochemicals including ATP during hypoxia, and contiguous glial-like type II cells which express purinergic P2Y2 receptors (P2Y2R). We previously showed that activation of P2Y2R on rat type II cells led to the opening of pannexin-1 (Panx-1) channels, which acted as conduits for the further release of ATP. More recently, we considered the possibility that other CB neuromodulators may have a similar paracrine role, leading to the activation of type II cells. Here, we examine the evidence that angiotensin II (ANG II), endothelin- (ET-1), and muscarinic agonists (e.g. acetylcholine, ACh) may activate intracellular Ca^{2+} signals in type II cells and, in the case of ANG II and ACh, Panx-1 currents as well. Using ratiometric Ca^{2+} imaging, we found that a substantial population of type II cells responded to 100 nM ANG II with a robust rise in intracellular Ca^{2+} and activation of Panx-1 current. Both effects of ANG II were mediated via AT_1 receptors (AT_1Rs) and current activation could be inhibited by the Panx-1 channel blocker, carbenoxolone (CBX; 5 μM). Additionally, low concentrations of ET-1 (1 nM) evoked robust intracellular Ca^{2+} responses in subpopulations of type II cells. The mAChR agonist muscarine (10 μM) also induced a rise in intracellular Ca^{2+} in some type II cells, and preliminary perforated-patch, whole-cell recordings revealed that ACh (10 μM) may activate Panx-1-like currents. These data suggest that paracrine activation of type II cells by endogenous neuromodulators may be a common feature of signal processing in the rat CB.

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ATP • Gliotransmission • Angiotensin II • Endothelin-1 • Acetylcholine • Pannexin-1 channels

5.1 Introduction

In the carotid body (CB), **g**lial **f**ibrillary **a**cidic **p**rotein (GFAP)-positive glial-like type II cells occur in intimate association with chemoreceptor (type I) cells in the ratio of approximately 1:4 (McDonald 1981). The elongated cell bodies of type II cells extend cytoplasmic processes that ensheath type I clusters, suggesting paracrine interactions may occur between these two cell types (Tse et al. 2012; Nurse and Piskuric 2013). So far, most of the paracrine mechanisms investigated in the CB have targeted the chemoreceptor type I cells, which express a variety of G-protein coupled receptors for endogenous neuromodulators (Kumar and Prabhakar 2012; Nurse 2010; Nurse and Piskuric 2013). By comparison, the potential role of paracrine signaling via glial-like type II cells has been less well studied (Tse et al. 2012). Glial cells in the central nervous system may monitor, respond to, and participate in synaptic activity by releasing neuroactive substances in a process known as 'gliotransmission' (Eroglu and Barres 2010; Parpura et al. 2012). Zhang et al. (2012) proposed that within the rat CB, type I cells, sensory nerve endings, and glial-like type II cells may communicate within a tripartite synaptic complex, where sensory transmission is modulated, in part by purinergic mechanisms. In support of this schema, activation of purinergic P2Y2 receptors on type II cells led to the opening of gap-junction-like, pannexin 1 (Panx-1) channels and release of ATP (Zhang et al. 2012). Given the central role of ATP as a key excitatory neurotransmitter released from type I cells during chemoexcitation (Zhang et al. 2000; Nurse 2010; Nurse and Piskuric 2013), the study of Zhang et al. (2012) suggested a novel pathway whereby paracrine stimulation of type II cells during chemoexcitation might help boost the excitatory signal, ATP.

In a more recent search for other CB neurochemicals that might be capable of similarly stimulating type II cells, we tested the effects of the vasoactive neuropeptides, angiotensin II (ANG II) and endothelin-1 (ET-1), as well as the putative CB neurotransmitter, acetylcholine (ACh). In agreement with an earlier preliminary report (Tse et al. 2012), we found that type II cells of the rat CB can respond to both ANG II (Murali et al. 2014) and muscarinic agonists, leading to a rise in intracellular Ca^{2+} concentration. A novel finding was that low, nanomolar concentrations of ET-1 can also elicit robust intracellular Ca^{2+} responses in a subpopulation of type II cells and that, similar to ATP and ANG II (Murali et al. 2014), the effects of ACh may lead to the activation of Panx-1 channels.

5.2 Materials and Methods

5.2.1 Cell Culture

Dissociated rat CBs were cultured as previously described (Zhang et al. 2000, 2012). Briefly, CBs were removed from juvenile (P9-14) rats and placed in ice-cold L15 medium. All procedures for animal handling were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC) and institutional guidelines. The excised CBs were enzymatically dissociated for 1 h, then mechanically dissociated with forceps. The cells were then triturated and plated on culture dishes with modified F-12 medium.

5.2.2 Fura-2 Ratiometric Ca^{2+} Imaging

Intracellular Ca^{2+} measurements were performed as previously described (Piskuric and Nurse 2012; Murali et al. 2014). Briefly, CBs were loaded with 2.5 μM fura-2 diluted in standard bicarbonate-buffered solution (BBS) for 20 min at 37 °C, and subsequently washed for ~15 min to remove free dye. The dish was placed on the stage of an inverted microscope and perfused with BBS buffered with 95 % air/5 % CO_2 at 37 °C.

5.2.3 Electrophysiology

Nystatin perforated-patch, whole cell-recording was used to monitor ionic currents in type II cells as previously described (Zhang et al. 2012). All recordings were carried out at ~35 °C and the cells were perfused with standard BBS. Agonists (ANG II, ET-1, ACh) were applied by a 'fast perfusion' system utilizing a double-barreled pipette assembly as previous described (Zhang et al. 2012).

5.2.4 Solutions and Drugs

For calcium imaging, the BBS contained (in mM): $NaHCO_3$, 24; NaCl, 115; glucose, 5; KCl, 5; $CaCl_2$, 2 and $MgCl_2$, 1, at 37 °C and the pH was kept at 7.4 by bubbling the solution with a 95 % air/5 % CO_2 gas mixture. For electrophysiology, the BBS contained (in mM): $NaHCO_3$, 24; NaCl, 115; glucose, 10; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1, and sucrose, 12. The pipette solution contained (mM): potassium gluconate, 115; KCl, 25; NaCl, 5; $CaCl_2$, 1; Hepes, 10, and nystatin 200 $\mu g.ml^{-1}$; at pH 7.2.

5.3 Results

Experiments were carried out on isolated 'solitary' type II cells to minimize cross-talk arising from neurosecretion by neighbouring type I cells. In Ca^{2+} imaging experiments, type II cells were identified by the presence of an increase in intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$) during stimulation with UTP, a selective P2Y2 receptor agonist (Xu et al. 2003; Piskuric and Nurse 2012; Tse et al. 2012; Zhang et al. 2012). In these experiments, the absence of cross-talk was indicated when type II cells failed to elicit a $\Delta[Ca^{2+}]_i$ response during perfusion with the depolarizing stimulus high K^+, which stimulates neurosecretion from type I cells (Buttigieg and Nurse 2004; Livermore and Nurse 2013). In voltage clamp experiments, solitary type II cells were identified by their elongated morphology and electrophysiological profile (Duchen et al. 1988; Zhang et al. 2012; Murali et al. 2014).

5.3.1 Angiotensin II (ANG II) Stimulates a Rise in $[Ca^{2+}]_i$ and Activates Panx-1 Currents in Type II Cells via AT_1 Receptors

It is well established that ANG II elicits a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in at least a subpopulation of rat type I cells (Fung et al. 2001). In a recent study (Murali et al. 2014), we confirmed this finding and further showed that type II cells also respond to ANG II (see also, Tse et al. 2012), and with an even larger increase in $[Ca^{2+}]_i$. Figure 5.1a illustrates an example where ANG II (100 nM) elicited a Ca^{2+} response in both a type I and type II cell in the same culture. Interestingly, in the presence of suramin (100 μM) which should block any cross-talk from type I to type II cells mediated via P2Y2R stimulation, the $\Delta[Ca^{2+}]_i$ response to ANG II was unaffected as exemplified in Fig. 5.1a; summary data from one experimental series supporting this point are shown in Fig. 5.1b (n = 3 dishes; 30–100 cells sampled per dish). These data support a direct interaction between ANG II and AT receptors on type II cells, without type I cell involvement. Results from a recent study of >500 UTP-sensitive type II cells revealed that ~75 % of them were also sensitive to ANG II (Murali et al. 2014). The intracellular Ca^{2+} signal elicited by

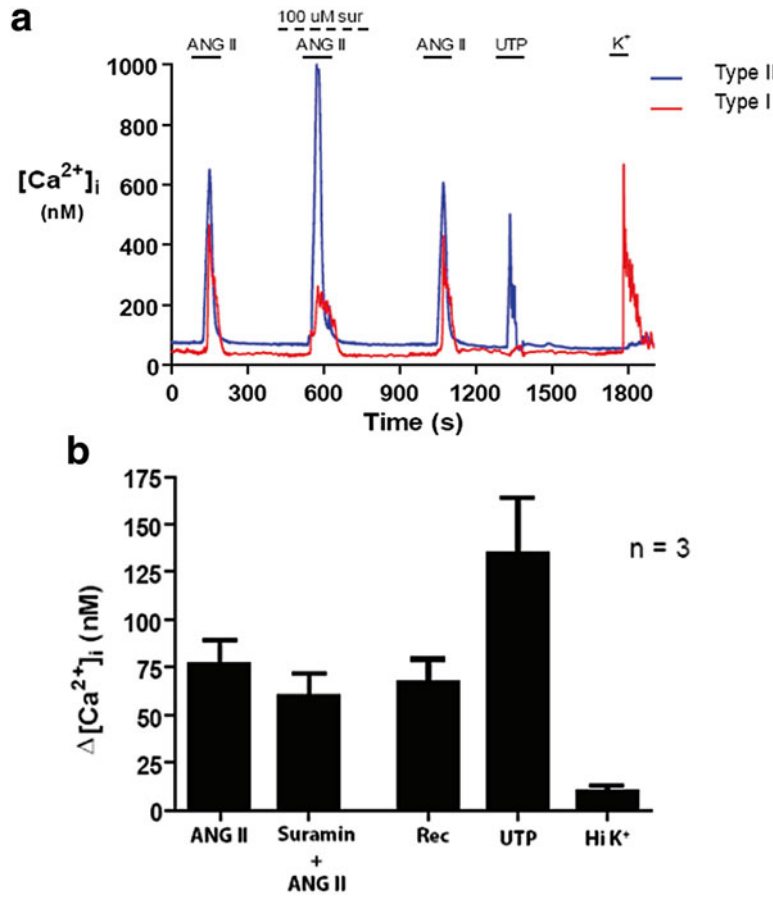

Fig. 5.1 Angiotensin II (ANG II) elicits intracellular Ca^{2+} responses in both type I and type II cells. In (**a**) ANG II-induced Ca^{2+} responses occur with similar latencies in type I and type II cells. Suramin (100 μM) failed to block ANG II-evoked Ca^{2+} responses in type II cells (**a**, **b**), suggesting these responses were not secondary to release of ATP from type I cells (n = 3 dishes; 30–100 cells sample per dish). Note UTP and high K^+ (30 mM) selectively elicit Ca^{2+} responses in type II and type I cells respectively

ANG II in type II cells arose from intracellular stores, and was mediated via AT_1 receptors (AT_1R) because it could be reversibly abolished by the specific AT_1R blocker, 1 μM losartan (Murali et al. 2014).

To determine whether stimulation of AT_1R by ANG II also led to the activation of Panx-1 currents in type II cells, similar to P2Y2R activation by ATP or UTP (Zhang et al. 2012), we used voltage clamp. Indeed, at a holding potential of −60 mV, ANG II (100 μM) induced an inward current in type II cells that was reversibly abolished by 5 μM carbenoxolone (CBX), a selective Panx-1 channel blocker (Ma et al. 2009; Murali et al. 2014). This inward current was also reversibly abolished by losartan (1 μM), confirming that both the rise in $[Ca^{2+}]_i$ and activation of Panx-1 current were mediated via AT_1R in type II cells (Murali et al. 2014).

5.3.2 Endothelin 1 (ET-1) Stimulates a Rise in $[Ca^{2+}]_i$ in Type II Cells

In addition to ANG II, endothelin 1 (ET-1) is another vasoactive neuropeptide expressed in rat CB type I cells, and its expression is upregulated during whole animal exposure to chronic hypoxia

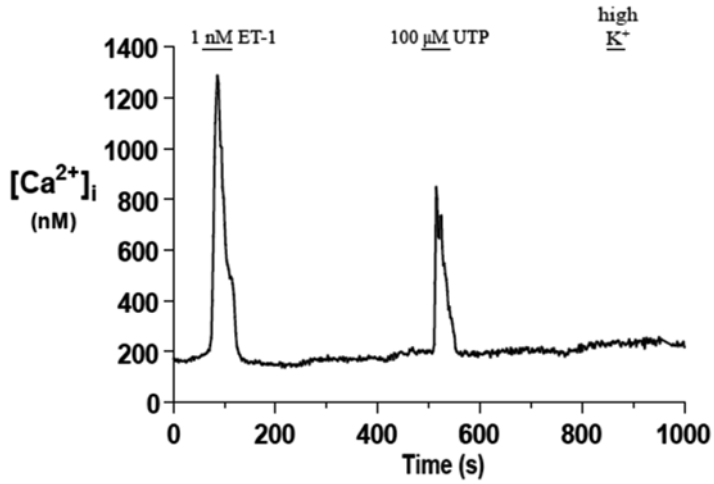

Fig. 5.2 Effects of endothelin-1 (ET-1) on intracellular Ca^{2+} in carotid body type II cells. The sample trace shows a rise in $[Ca^{2+}]_i$ in response to 1 nM ET-1 and 100 μM UTP in a type II cell. The ET-1 response was typical of 60/100 cells that were sensitive to UTP

(Chen et al. 2002a, b). Moreover, there is recent evidence that release of ET-1 from type I cells can stimulate proliferation of CB cells by acting mainly at ET(B) receptors on $GFAP^+$, glial-like progenitors (Platero-Luengo et al. 2014). Though in the latter study, weak expression of ET(A) receptors was also found on a subpopulation of $GFAP^+$ cells, the possibility is raised that CB type II cells may express ET(A) and/or ET(B) receptors. Indeed, as exemplified in Fig. 5.2, exposure of type II cells to a relatively low dose of ET-1 (1 nM) caused a robust rise in $[Ca^{2+}]_i$, comparable to that seen with much higher concentrations of ANG II (100 nM) and UTP (100 μM). The mean $\Delta[Ca^{2+}]_i$ response induced by 1 nM ET-1 in solitary type II cells was 96.7 ± 9.8 nM (n = 60 cells). Future experiments will determine which subtype(s) of ET-receptors mediate(s) these $\Delta[Ca^{2+}]_i$ responses, as well as the intracellular signaling pathway involved.

5.3.3 ACh Mobilizes Ca^{2+} and Activates Panx-1-Like Currents in Type II Cells

In a preliminary study, stimulation of muscarinic ACh receptors (mAChRs) on rat type II cells led a rise in intracellular Ca^{2+} (Tse et al. 2012). Figure 5.3a confirms that stimulation of mAChR with 10 μM muscarine caused a rise in $[Ca^{2+}]_i$ in the same type II cell that was responsive to 100 μM UTP. We found that ~53 % (149/280 cells) of UTP-sensitive type II cells were also sensitive to 10 μM muscarine; the mean $\Delta[Ca^{2+}]_i$ response induced by muscarine was ~36 nM in type II cells. This Ca^{2+} response to mAChR stimulation appeared much smaller in comparison with responses to ATP/UTP (Zhang et al. 2012) and ANG II (see above). To determine whether mAChR stimulation can lead to Panx-1 channel opening we used voltage clamp. In preliminary studies, rapid perfusion of 10 μM ACh resulted in the activation of Panx-1-like currents in a subpopulation of type II cells. An example is shown in Fig. 5.3b, where both ATP (Fig. 5.3b1) and ACh (Fig. 5.3b2) activated Panx-1-like currents in the *same* type II cell; recordings are typical of n = 5 cells.

5.4 Discussion

In this study we consider the evidence for paracrine signaling in the rat carotid body involving the action of neurochemicals at glial-like type II cells. Previous studies demonstrated that type II cells express P2Y2 receptors (P2Y2R) which,

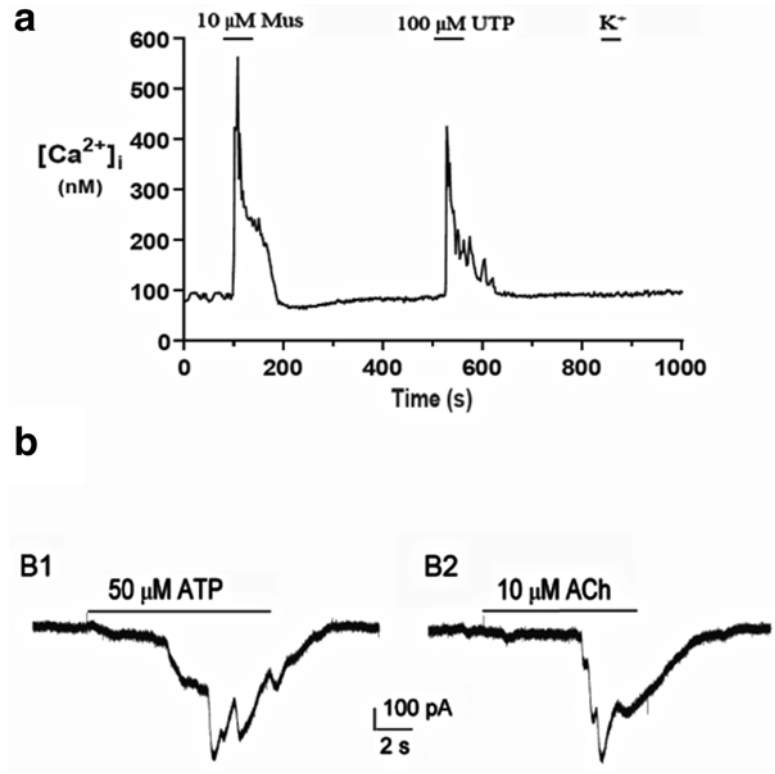

Fig. 5.3 Muscarine (Mus) elicits Ca^{2+} responses and acetylcholine (ACh) activates pannexin-1 (Panx-1) currents in rat carotid body type II cells. Sample trace shows a rise in Ca^{2+} evoked by 10 µM Mus and 100 µM UTP in a type II cell (**a**). The response to Mus was present in 53 % (150/280) of UTP-sensitive type II cells. Both ATP (**b1**) and ACh (**b2**) activated Panx-1-like currents in the same cell (n = 5)

when stimulated by agonists such as ATP and UTP, elicited a rise in intracellular Ca^{2+} (Xu et al. 2003; Tse et al. 2012; Zhang et al. 2012). Moreover, activation of this signaling pathway led to a Ca^{2+}-dependent opening of Panx-1 channels that allowed further release of ATP (Zhang et al. 2012; Murali et al. 2014). In the study by Zhang et al. (2012), stimulation of type II cells alone with UTP could trigger release of ATP that was sufficient to excite afferent petrosal neurons in co-culture. Together, these studies suggest that purinergic signaling pathways in type II cells may contribute to sensory processing in the carotid body.

Recent evidence, including preliminary data described in the present study, point to the type II cell as a likely target for other neurochemicals released by type I cells during chemotransduction. For example, the vasoactive neuropeptides angiotensin II (ANG II) (Tse et al. 2012; Murali et al. 2014) and endothelin-1 (ET-1) (this study), which are known to be expressed in chemoreceptor type I cells, evoke a robust rise in intracellular Ca^{2+} in type II cells. Similar to ATP acting via P2Y2R, the signaling pathway activated by ANG II led to a Ca^{2+}-dependent opening of Panx-1 channels (Murali et al. 2014). Further studies are required to determine whether ET-1 can act similarly. Nevertheless, our preliminary data suggest that another neurochemical ACh, whose cellular localization and role in CB physiology remains controversial (Nurse 2010; Nurse and Piskuric 2013), is capable of eliciting a rise in intracellular Ca^{2+} and Panx-1 channel opening in at least a subpopulation of type II cells. This pathway involves activation of G-protein coupled mAChR

receptors (Tse et al. 2012; this study). Taken together, these studies demonstrate that several CB neurochemicals synthesized in type I cells may trigger intracellular Ca^{2+} responses in type II cells. These paracrine pathways may also lead to the opening of Panx-1 channels and release of ATP, a 'gliotransmitter' capable of exciting afferent petrosal neurons (Zhang et al. 2012). However, it still remains to be formally demonstrated that these proposed signaling pathways do in fact play a significant role in synapse integration in the CB during chemotransduction.

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