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DISTRIBUTION AND LOCALISATION OF Gα PROTEINS IN THE ROSTRAL VENTROLATERAL MEDULLA OF NORMOTENSIVE AND HYPERTENSIVE RATS: FOCUS ON CATECHOLAMINERGIC NEURONS

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Abstract—About 860 G-protein-coupled receptors (GPCRs) mediate their actions via heterotrimeric G-proteins. Their activation releases Gα from Gβλ subunits. The type of Gα subunit dictates the major signalling proteins involved: adenylyl cyclase, PLC and rhoGEF. The rostral ventrolateral medulla (RVLM), containing the rostral C1 (rC1) cell group, sets and maintains the tonic and reflex control of blood pressure and a plethora of inputs converge onto these neurons. We determined the relative abundance of 10 Gα subunit mRNAs, representing the four major families, within the RVLM, using quantitative RT-PCR. *In situ* hybridisation (ISH) combined with immunohistochemistry (IHC) was used to quantify and compare this expression in rC1 with that in the A1 and A5 cell groups. The relative abundance of Gα subunit mRNAs and a comparison of gene expression levels were quantitatively determined in normotensive and hypertensive rat strains. All 10 Gα mRNAs were detected in the RVLM of Sprague–Dawley (SD) rats with relative abundance such that Gαs > Gαi2 > Gαo > Gαq > GαL > Gα11 > Gαi3 > Gαi1 > Gα12 > Gα13. The high abundance of Gα mRNAs signalling via adenylyl cyclase indicates the importance of associated GPCRs. Within the rC1 and A1 groups similar differential Gα mRNA expression profiles were seen with Gαs being found in all rC1 cells, Gα11 absent and Gαi3 rarely expressed. Thus functionally distinct subgroups exist within the rC1 and A1 cell groups as differing distributions of Gα subunits must reflect the array of GPCRs that influence their activity. In contrast, all A5 cells expressed all Gα mRNAs suggesting a functionally homogeneous group. When the 10 Gα mRNAs of the RVLM in spontaneously hypertensive rats (SHR) were compared quantitatively to Wistar-Kyoto (WKY), only Gαs and Gα12 were significantly elevated. However when the expression in normotensive SD and WKY was compared with SHR no significant differences were evident. These findings demonstrate a range of GPCR signalling capabilities in brainstem neurons important for homeostasis and suggest a prominent role for signalling via adenylyl cyclase.

Key words: rostral ventrolateral medulla, rostral C1, A1, A5, G-proteins, spontaneously hypertensive rat.

Heterotrimeric G-proteins belong to the family of guanine nucleotide-binding proteins that relay signals from G-protein-coupled receptors (GPCRs) to downstream intracellular effector proteins including adenylyl cyclase, phospholipases and rhoGEF (Gilman, 1987; Offermanns, 2003; Wettschureck and Offermanns, 2005). Once the GPCR is activated the Gα subunit exchanges its bound GDP for GTP allowing dissociation of the Gβγ subunits and permitting these entities to transduce signals independently. Traditionally G-proteins have been divided into four major families based on the α-subunit sequence identity which selects their regulation for specific effector proteins: Gαs/L family stimulates adenylyl cyclase, Gαi/o family inhibits adenylyl cyclase, Gαq/11 family stimulates phospholipase Cβ and Gα12/13 family activates rhoGEF and other downstream effectors (Simon et al., 1991; Downes and Gautam, 1999; Wettschureck and Offermanns, 2005). In human, Gα subunits are the product of 16 genes defining about 20 different proteins including splice variants (Downes and Gautam, 1999). An array of other effectors, in particular ion channels, can also be directly activated (Brown and Birnbaumer, 1988).

Within the brainstem, the rostral part of the ventrolateral medulla (RVLM) is an important site controlling autonomic and other functions. It contains presympathetic neurons that regulate the heart, blood vessels and adrenal gland, neurons that control the rhythm and pattern of respiration as well as motor and premotor neurons (Pilowsky and Goodchild, 2002; Guyenet, 2006; Rybak et al., 2007). A large number of neurotransmitters, neuropeptides and other neuromodulators acting at GPCRs in the RVLM likely regulate these functions and some have been identified. For example, acetylcholine acting via muscarinic receptors increases blood pressure (Padley et al., 2007) whereas somatostatin (SST) decreases blood pressure (Burke et al., 2008). Cardiorespiratory reflex function can be modified specifically by a range of

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Abbreviations: AP, alkaline phosphatase; Ct, cycle threshold; DIG, digoxigenin; GPCRs, G-protein-coupled receptors; IHC, immunohistochemistry; ISH, *in situ* hybridisation; LC, locus coeruleus; NSE, neuron specific enolase; rC1, rostral C1; RVLM, rostral ventrolateral medulla; SD, Sprague–Dawley; SHR, spontaneously hypertensive rats; SN, substantia nigra; SST, somatostatin; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase immunoreactive; VTA, ventral tegmental area; WKY, Wistar-Kyoto.

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agents which act at GPCRs in the ventrolateral medulla (Pilowsky et al., 2008). For example, activation of postsynaptic muscarinic receptors in the RVLM enhances baroreflex function (Padley et al., 2007). Functionally specific populations of neurons do not have phenotypes although substantial evidence indicates that rostral C1 (rC1) neurons participate in cardiorespiratory reflex regulation (Schreihofer and Guyenet, 2000; Madden et al., 2006) and may play a role in regulating sympathetic vasomotor tone (Marina et al., 2011).

The distribution and/or localisation of some Gα subunits that mediate GPCR function have been described in some higher brain regions (Worley et al., 1986; Asano et al., 1988; Largent et al., 1988; Mailleux et al., 1992; McFarlane-Anderson et al., 1992; Milligan, 1993; Khan and Gutierrez, 2004) however the lower brainstem has rarely been investigated. Within each Gα family there is good evidence of differential tissue expression (Okuhara et al., 1996). It is not known if neurons in the lower brainstem contain distinct complements of Gα subunits or whether some Gα subunits are ubiquitously distributed or absent.

Alterations of Gα subunit levels or function have been associated with various diseases or conditions (McFarlane-Anderson et al., 1992; Fu et al., 1994a; Avissar et al., 1997) including high salt challenge and cardiovascular disease: high salt evoked down regulation of Gαq in the hypothalamic paraventricular nucleus of untreated rats but not Dahl salt-sensitive rats (Wainford and Kapusta, 2010). Gαi is upregulated in the heart, in heart failure (Feldman et al., 1989) and function of the protein may be altered in hypertension (McLellan et al., 1993a,b; Fu et al., 1994b; Siffert, 2005). An increase in Gαi mRNA expression, specifically Gαi2 and Gαi3, in the aorta and heart, was seen in spontaneously hypertensive rats (SHR) and this increase is present prior to the development of hypertension (Anand-Srivastava et al., 1991; Fu et al., 1994b). It has been suggested that enhanced concentrations of angiotensin or endothelins in vascular smooth muscle cells which transactivate epidermal growth factor receptors to enhance MAP kinase evoke the increase in Gαi levels (Sandoval et al., 2011). In contrast, reductions in Gαi3 protein were found in vascular tissue from the Milan hypertensive strain and Gαs levels were also reduced (Clark et al., 1993). The activity of sympathetic nerves innervating the vasculature is elevated in hypertensive rats and humans and such activity is regulated by the RVLM. Furthermore, altering the expression of GPCRs or their related signalling pathways, in the RVLM, influences blood pressure (Seyedabadi et al., 2001; Allen, 2011). Thus, whether alterations in Gα subunit mRNA distribution were present in RVLM in hypertension is of interest.

Thus, the aims of this project were to determine the distribution of 10 major Gα subunit mRNAs, representing the four major families, in the RVLM. We first determined their localisation within a defined RVLM cell population, the rC1 neurons, in Sprague–Dawley (SD) rats and compared this with distributions in two other catecholaminergic cell groups subserving allied but different homeostatic functions, the A5 and A1 cell groups. A1 cells primarily regulate blood volume (Day, 1989) whereas A5 neurons appear to mediate the visceral response to acute hypoxia (Kanbar et al., 2011). We then determined, and compared quantitatively, the mRNA expression in the RVLM of three rat strains: normotensive SD, normotensive Wistar-Kyoto (WKY) rats and SHR.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the Macquarie University Animal Ethics Committee and were carried out following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Experiments were performed on adult male SD rats ($n = 9$ (3 for *in situ* hybridisation (ISH) and 6 for quantitative PCR; 400–650 g)), and 20–23 week old WKY rats ($n = 6$) and SHR ($n = 6$) that were obtained from the Animal Resource Centre, Perth, WA, Australia and housed at the Central Animal House Facility, Macquarie University. The systolic blood pressures were measured using tail cuff plethysmography in SHR (198 ± 4 mm Hg) and WKY (132 ± 5 mm Hg).

Riboprobe and antibody characterisation

Table 1 describes the oligonucleotide primer sequence, riboprobe length and the GeneBank accession number for the 10 Gα subunit mRNAs investigated here. Non-radioactive sense and antisense RNA probes were *in vitro* transcribed (T7-Flash Kit, Epicentre Biotechnologies, Madison, WI, USA) from PCR-generated cDNA template that was amplified from primers containing SP6 and T7 RNA polymerase recognition sites. Riboprobes, incorporated with digoxigenin-11-UTP (Roche Applied Science), were custom synthesized and validated as described previously (Li et al., 2003; Padley et al., 2007). ISH of Gα subunit riboprobes was performed in conjunction with fluorescence immunohistochemistry detection of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis. Monoclonal mouse anti-TH antibody (Sigma clone TH-2, cat#T1299) recognises the epitope present on the N-terminal region of the rodent (≈60 kDa) TH. Sheep anti-digoxigenin (DIG) antibody from Roche Applied Science (alkaline phosphatase (AP) conjugated cat#11093274910) followed by enzymatic detection was used to visualise hybridised riboprobe.

Combined ISH and immunohistochemistry (IHC)

Rats were deeply anaesthetised with sodium pentobarbital (80 mg/kg i.p.) and perfused transcardially with 300 ml of ice-cold heparinised saline followed by 300 ml of ice cold 4% PFA/0.1 M PB, pH 7.4 (Sigma–Aldrich, Australia). The brainstem was isolated and fixed overnight in the same fixative at 4 °C prior to sectioning. Coronal brainstem sections (40 μm thick) were cut on a vibrating microtome (VT1299S, Leica Microsystems, North Ryde, NSW, Australia) and collected sequentially into pots containing PBS with 0.1% Tween 20 (Sigma–Aldrich, Australia). Free-floating tissues' sections were processed for ISH using riboprobes (Table 1) combined with fluorescence IHC visualisation of TH using previously described methods (Stornetta et al., 2001; Padley et al., 2007; Farnham et al., 2008).

Briefly, tissue sections were incubated in prehybridisation buffer (50% formamide, 5× SSC, pH 7.0, 250 μg/ml herring sperm DNA, 100 μg/ml yeast tRNA, 100 μg/ml heparin, 5% dextran sulphate, 1× Denhardts solution, 0.1% Tween 20) for 2 h at 37 °C and then at 58 °C for 1 h. This was followed by hybridisation with a G-protein α subunit riboprobe (final concentration 100–200 ng/ml). Hybridisation occurred for 12–18 h at 58 °C with gentle agitation. After post-hybridisation washes, the tissue was