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EFFECTS OF THE CALCIUM-REGULATING GLYCOPROTEIN HORMONE STANNIOCALCIN-1 WITHIN THE NUCLEUS OF THE SOLITARY TRACT ON ARTERIAL PRESSURE AND THE BARORECEPTOR REFLEX

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Abstract—Receptors for the calcium-regulating glycoprotein hormone stanniocalcin-1 (STC-1) have been found within the CNS and whether these receptors exist within the nucleus of the solitary tract (NTS), and their possible role in the regulation of arterial pressure (AP) is unknown. Experiments were done in the rat to: (1) map the distribution of STC-1 receptors throughout NTS using *in situ* ligand binding that uses a stanniocalcin-alkaline phosphatase (STC-AP) fusion protein; (2) determine whether protein and gene expression for STC-1 exists within NTS using immunohistochemistry, Western blot and real time qPCR; (3) determine the effect of microinjection of STC-1 into NTS on AP and the baroreflex. Cells exhibiting STC-1 binding sites were found mainly within the caudal medial (Sm), gelantinous and commissural subnuclei of NTS. Cells containing STC-1 immunoreactivity were found to overlap those regions of NTS that contained STC-1 receptors. STC-1 protein and gene expression were also found within caudal NTS. In chloralose-urethane–anesthetized rats, microinjections of STC-1 (1.76–176 nM; 20 nl) into the caudal Sm elicited a dose-related decrease in AP. In contrast, injections of a nonbioactive form of STC-1 (STC-1+guanosine 5′-triphosphate [GTP]), the vehicle (0.9% saline), or GTP alone did not elicit cardiovascular responses. Additionally, injection of STC-1 into Sm potentiated the AP responses to electrical stimulation of the ipsilateral aortic depressor nerve. Finally, bilateral injection of STC-1 primary antiserum (1:1000; 100 nl) into Sm elicited a long lasting increase in AP, whereas microinjection of heat inactivated STC-1 antiserum did not alter AP. Taken together these data suggest that endogenous STC-1 signaling in NTS is involved in regulating the excitability of neurons that normally function as components of the baroreceptor reflex controlling AP.

Key words: stanniocalcin-1 receptors, stanniocalcin-1 immunohistochemistry, stanniocalcin-1 protein and gene expression, cardiovascular responses, heart rate, baroreflex.

Stanniocalcin (STC) was first identified in the corpuscle of Stannius, a gland mistakenly assumed to be the adrenal gland attached to the kidney of fish (for reviews see Gerritsen and Wagner, 2005; Wagner and Dimattia, 2006; Yoshiko and Aubin, 2004). STC, a 50-kDa disulfide linked glycoprotein dimer of identical subunits (Olsen et al., 1996), has been shown to reduce calcium uptake in gill and gut epithelial cells and increase the reabsorption of phosphate in kidney tubules (Fenwick, 1974b; Fontaine, 1964). This effect of STC is thought to be mediated through activation of calcium-sensing receptors that respond to increased serum calcium levels (Fenwick, 1974a; Radman et al., 2002).

A mammalian homologue of STC, stanniocalcin-1 (STC-1) from human kidney proteins (Wagner et al., 1995) has been recently cloned (Gerritsen and Wagner, 2005). Human STC-1 shows high homology (73%) to the fish hormone and is widely expressed in a variety of tissues such as adipose, kidney, heart, skeletal muscle, lung, ovaries, and brain (Gerritsen and Wagner, 2005; Olsen et al., 1996; Zhang et al., 1998). In rats, injections of recombinant human STC-1 (hSTC-1) stimulate renal tubular phosphate reabsorption, and the primary mechanism of action appears to be through the activation of sodium-phosphate co-transporters (Olsen et al., 1996; Wagner et al., 1997). In addition, application of STC-1 to the serosal surface of rat or pig duodenal mucosa stimulates intestinal absorption of phosphate and decreases that of calcium (Madsen et al., 1998). These effects of STC-1 appear to mirror those in homologous fish organ systems (Gerritsen and Wagner, 2005).

In fish and mammals, STC-1 expression has been observed in the brain (McCudden et al., 2001; Zhang et al., 1998). Immunoreactive cells to STC-1 have been found

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Abbreviations: ADN, aortic depressor nerve; ap, area postrema; AP, arterial pressure; cc, central canal; com, commissural subnucleus of the nucleus of the solitarytract complex; cp, choroid plexus; Cu, cuneate nucleus; DMV, dorsal motor nucleus of the vagus; GR, nucleus gracilis; GTP, guanosine 5′-triphosphate; HBHA, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HR, heart rate; hSTC-1, recombinant human stanniocalcin-1; MAP, mean arterial pressure; mRNA, messenger ribonucleic acid; NBT-BCIP, nitro-blue tetrazolium chloride and 5-bromo 4-chloro 3′indolyphosphate p-toluidine salt; NTS, nucleus of the solitary tract; PBS, phosphate-buffered saline; qPCR, real-time quantitative reverse transcriptase polymerase chain reaction; Sc, central subnucleus of the nucleus of the solitary tract complex; Sdl, dorsolateral subnucleus of the nucleus of the solitary tract complex; SDS, sodium dodecyl sulfate; SInt, intermediate subnucleus of the nucleus of the solitary tract complex; Sg, nucleus gelatinous of the nucleus of the solitary tract complex; Sl, lateral subnucleus of the nucleus of the solitary tract complex; Sm, medial subnucleus of the nucleus of the solitary tract complex; Sni, interstitial subnucleus of the nucleus of the solitary tract complex; St, solitary tract; STC, stanniocalcin; STC-1, stanniocalcin-1; STC-AP, stanniocalcin-alkaline phosphatase fusion protein; Svl, ventrolateral subnucleus of the nucleus of the solitary tract complex; TTBS, Tween Tris-buffered saline; 4V, fourth ventricle; 12M, hypoglossal nucleus.

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within the parietal cortex, dentate nucleus and cerebellar cortex of human brains (Zhang et al., 1998), as well as in the hippocampus and prefrontal cortex of mice (Long et al., 2003), and the subfornical organ of rats (unpublished data). In addition, the epithelial cells of the choroid plexus synthesize and express STC-1 in humans (Franzén et al., 2000). Although STC-1 has been shown to be expressed in various areas of the brain, there are no data suggesting a function for STC-1 in the regulation of neuronal function, except that it may act as an anti-apoptotic agent in cortical neurons (Zhang et al., 1998, 2000).

Lukovic et al. (1989) found that changing the ratio of sodium to calcium ions available to neurons within the nucleus of the solitary tract (NTS), the primary site of termination of cardiovascular afferent fibers (Ciriello et al., 1994), induced hypotension, and bradycardia. This finding was supported by the observation that the cardiovascular responses were likely due to the increased excitability of cells in NTS following the removal of extracellular calcium ions or blockage of calcium entry into the cells (Higuchi et al., 1986). One of STC-1 functions is to maintain cellular calcium homeostasis (Ishibashi and Imai, 2002; Wagner and Dimattia, 2006). STC-1 has been shown to regulate cytosolic calcium levels by increasing mitochondrial uptake of the calcium through a uniporter-dependent mechanism (Ellard et al., 2007; McCudden et al., 2002). In addition, STC-1 has been shown to act like an L-type calcium channel blocker in the membrane of myocardial cells (Sheikh-Hamad et al., 2003). Therefore, it is possible that STC-1 may function to alter the excitability of NTS neurons that are involved in the control of arterial pressure (AP) and heart rate (HR).

This study investigates whether STC-1 contributes to the function of NTS in cardiovascular regulation. As a complete mapping of the NTS region containing STC-1 binding sites is not available, experiments were initially done to identify cells within the dorsal medial medulla that expressed STC-1 binding sites using *in situ* ligand binding, which utilizes a stanniocalcin-alkaline phosphatase fusion protein (STC-AP; Ratkovic et al., 2008). In a second series, cells containing STC-1 protein were immunohistochemically identified within the NTS region using an antiserum directed at the STC-1 protein (Haddad et al., 1996; Olsen et al., 1996). In a third series, protein levels and mRNA expression were determined within the NTS region using Western blot analysis (Turner et al., 2010), and quantitative reverse-transcriptase polymerase chain (qPCR) reaction (Turner et al., 2010), respectively. In a final series, experiments to investigate the effect of microinjection of STC-1 into NTS on AP, HR, and the baroreceptor reflex were done in the anesthetized rat. First, a systematic mapping of NTS was performed to identify cardiovascular responsive sites to discrete microinjection of STC-1. Second, experiments were done to investigate which components of the autonomic nervous system mediated these cardiovascular effects. Third, the STC-1 sequence also contains a functional nucleotide-binding cassette that when occupied by guanosine 5′-triphosphate (GTP) disrupts ligand-receptor interactions (Ellard et al., 2007), thus experiments were done to verify that the observed cardiovascular responses were due to STC-1 binding a functional receptor. To accomplish this, microinjections of STC-1 pre-incubated with GTP into NTS were made. Fourth, the effect of microinjecting STC-1 into NTS on the cardiovascular responses to electrical stimulation of the ipsilateral aortic depressor nerve, known to selectively mediate aortic baroreceptor afferent information (Sapru et al., 1981), was investigated. Finally, to determine whether an endogenous STC-1 signaling pathway exists within NTS, microinjections of a highly specific STC-1 antibody, so as to neutralize the putative, endogenous ligand, were made bilaterally into STC-1 responsive sites in NTS. These studies have shown that STC-1 receptors and STC-1 protein and mRNA are expressed within neurons of NTS. Furthermore, microinjections of STC-1 into NTS evoke sympathoinhibitory responses and facilitate the baroreceptor reflex, whereas blocking endogenous STC-1 results in an increase in AP.

EXPERIMENTAL PROCEDURES

General Procedure

Experiments were done in adult male Wistar rats ($n=67$; 300–450 g; Charles River Canada Inc., St.-Constant, QC, Canada). The minimal number of animals needed for statistical comparisons were used within these studies. All animals were housed under controlled conditions with a 12-h light/dark cycle. Food and water were available to all animals *ad libitum*. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

Histological localization of STC-1 receptors by *in situ* ligand Binding

Rats ($n=4$) were anesthetized using a mixture (4:1) of ketamine: domitor (0.1 ml/100 g, i.p.) and perfused transcardially with 300 ml of phosphate-buffered saline (PBS) at pH 7.4, followed by 500 ml of a PBS solution containing 4% paraformaldehyde. The brainstem was removed, fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 6 μm. *In situ* ligand binding was performed as previously described for the cellular localization of STC-1 receptors (McCudden et al., 2002; Ratkovic et al., 2008) on sections through the region of the dorsal medial medulla. The histological method employs a fusion protein of STC and human placental alkaline phosphatase (STC-AP). As previously described (Ratkovic et al., 2008), the tissue sections were dewaxed and dehydrated, equilibrated in Hanks' balanced salt solution containing 0.1% BSA (HBHA buffer; pH 7.4), and incubated with HBHA containing 2500 mU/ml STC-AP for 90 min. Slides were then washed in HBHA+0.1% Tween and treated for 30 s with HEPES fixative (20 mM HEPES, 60% acetone and 3% paraformaldehyde, pH 7.5). To inactivate endogenous alkaline phosphatase activity, slides were incubated in Higgin's detection buffer (pH 9.5) at 65 °C for 30 min. The receptor localization was visualized by the addition of nitro-blue tetrazolium chloride and 5-bromo 4-chloro 3′indolyl phosphate p-toluidine salt (NBT-BCIP) that resulted in formation of a brownish-purple precipitate. Sections were mounted onto glass slides, dehydrated and coverslipped for analysis using bright-field microscopy.

Control brainstem sections were incubated in alkaline phosphatase alone and processed using NBT-BCIP (Ratkovic et al., 2008). Under these conditions, no labeling was observed within