Stanniocalcin-1 in the subfornical organ inhibits the dipsogenic response to angiotensin II

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Moreau JM, Iqbal W, Turner JK, Wagner GF, Ciriello J. Stanniocalcin-1 in the subfornical organ inhibits the dipsogenic response to angiotensin II. Am J Physiol Regul Integr Comp Physiol 303: R921–R928, 2012. First published August 29, 2012; doi:10.1152/ajpregu.00057.2012.—Recently, receptors for the calcium-regulating glycoprotein hormone stanniocalcin-1 (STC-1) have been found within subfornical organ (SFO), a central structure involved in the regulation of electrolyte and body fluid homeostasis. However, whether SFO neurons produce STC-1 and how STC-1 may function in fluid homeostasis are not known. Two series of experiments were done in Sprague-Dawley rats to investigate whether STC-1 is expressed within SFO and whether it exerts an effect on water intake. In the first series, experiments were done to determine whether STC-1 was expressed within cells in SFO using immunohistochemistry, and whether protein and gene expression for STC-1 existed in SFO using Western blot and quantitative RT-PCR, respectively. Cells containing STC-1 immunoreactivity were found throughout the rostrocaudal extent of SFO. STC-1 protein expression within SFO was confirmed with Western blot, and SFO was also found to express STC-1 mRNA. In the second series, microinjections (200 nl) of STC-1, ANG II, a combination of the two or the vehicle were made into SFO in conscious, unrestrained rats. Water intake was measured at 0700 for a 1-h period after each injection in animals. Microinjections of STC-1 (17.6 or 176 nM) alone had no effect on water intake compared with controls. However, STC-1 not only attenuated the drinking responses to ANG II for about 30 min, but also decreased the total water intake over the 1-h period. These data suggest that STC-1 within the SFO may act in a paracrine/autocrine manner to modulate the neuronal responses to blood-borne ANG II. These findings also provide the first direct evidence of a physiological role for STC-1 in central regulation of body fluid homeostasis. body fluid homeostasis; thirst; circumventricular organs; calcium-regulating glycoprotein

STANNOICALCIN (STC) IS A GLYCOPROTEIN hormone first identified and characterized within bony fish corpuscles of Stannius, endocrine glands derived from renal tubular cells (18, 24, 29, 63, 64). These glands are thought to produce and release STC into the circulation in response to rising serum calcium levels (11, 18, 29, 64). The STC then acts on the gills and gut epithelial cells to reduce calcium uptake, while STC within the kidney causes increases in reabsorption of phosphate (11, 18, 24), a mechanism that likely aids in chelating excess extracellular calcium (18, 24). The net effect of these STC actions is the restoration of serum calcium levels (14, 18, 24, 62).

A mammalian homolog of STC has been identified that shares a 73% amino acid sequence homology with fish STC (41). Mammalian STC-1 is a disulfide-linked glycoprotein dimer of identical subunits (41), which is expressed in a variety of tissues, including heart, kidney, adipose tissue, skeletal muscle, lung, ovary, and brain (67–69). STC-1 functions in the cells of these tissues to maintain calcium homeostasis (24, 63). STC-1 regulates cytosolic calcium levels by binding to the mitochondria (35) and increasing mitochondrial uptake of the calcium through a unipporter-dependent mechanism (10). Additionally, STC-1 has been shown to act like an L-type calcium channel blocker in the membrane of myocardial cells (51).

Recently, we have shown that the receptor for STC-1 exists within the subfornical organ (SFO) of the rat brain (45), a circumventricular organ that lacks a functional blood-brain barrier (12, 13, 15, 19, 25). The role of STC-1 within the SFO is not known, although it may function in modulating intracellular calcium concentrations within neurons, which could affect their excitability (17, 67–69). The SFO has been suggested to be one of the most important structures within the forebrain laminae terminalis in the regulation of electrolyte and body fluid homeostasis (1, 3, 7, 12, 13, 25, 31, 36, 37). Electrical and chemical stimulation of SFO has been reported to elicit dipsogenic responses in mammals (13, 46). In addition, a recent study has suggested that the SFO may also function in the regulation of food intake (55).

Immunohistochemical and autoradiographic binding studies have demonstrated that the SFO is a major site within the central nervous system at which the octapeptide ANG II binds within the central nervous system (22, 38) and activates neuronal circuits involved in the regulation of fluid and sodium balance, and blood pressure (12, 13, 15, 25, 36, 37). ANG II AT1 receptors (AT1R) have been identified on neurons throughout the SFO (44, 47, 56) and are known to mediate the dipsogenic and autonomic responses to ANG II (53, 54). On the other hand, blockade of the ANG II receptors within the SFO inhibits the dipsogenic and blood pressure response induced by ANG II (13, 15, 25, 53). The neuronal depolarization of SFO neurons in response to endogenous ANG II is mediated by increases in intracellular calcium concentration, which can be inhibited by the specific blockade of the AT1R (17, 26, 48, 66).

As ANG II acts in the SFO to elicit drinking, this study was done to investigate the contribution of STC-1 in the SFO to the ANG II-induced drinking by the SFO. In the first series, STC-1 protein within the SFO was determined using Western blot analysis. In addition, STC-1 mRNA expression in the SFO was determined using the quantitative RT-PCR (qRT-PCR) (57, 58). Furthermore, cells expressing STC-1 in the SFO were then identified immunohistochemically using an antiserum directed at the STC-1 protein (21, 41, 65). Finally, the effect of
MATERIALS AND METHODS

Experiments were done in adult male Sprague-Dawley rats (250–400 g) obtained from Charles River Canada (Montreal, Canada). All animals were housed individually under controlled conditions with a 12:12-h light-dark cycle beginning at 0700. Food and water were available to all animals ad libitum, except as noted below. All experimental procedures were done in accordance with the guidelines for 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.

Tissue collection for Western blots and qRT-PCR. Rats (n = 8) were killed using suffocation by CO₂, and the brains were immediately removed and snap frozen. Three hundred-micrometer transverse frozen sections through the SFO region were cut in a Bright’s cryostat, and the area containing SFO was punched-out using a 1-mm (internal diameter) metal tubing needle attached to a 1-cc syringe. The sections from which the punches were taken were then thaw-mounted onto double-gelatinized slides and stained with thionin for histological verification that the punched-out area was localized to the SFO. In addition, as the cerebellum is another extracortical central area that is suggested to express STC-1 protein (69), similar punchouts were taken of the cerebellar cortex. Furthermore, as previous studies have shown that the kidney expresses STC-1 mRNA (57, 59), for the qRT-PCR studies, small 3-mm³ samples of the kidney cortex were taken for comparisons of gene expression with the SFO and the cerebellum.

Western blot. For Western blot analysis, the SFO and cerebellum samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% phenylmethylsulfonyl fluoride) using an electric pestle homogenizer. Samples were then centrifuged at 14,000 rpm for 10 min to remove any remaining precipitate. Protein concentrations of the clear supernatant were standardized using the Bio-Rad Dₖ protein assay kit as per manufacturers’ instructions.

SDS-PAGE was carried out using a 10% discontinuous polyacrylamide gel (32), followed by Western blot analysis, as previously described (6, 42, 57, 58). For each sample, 70 μg of protein was loaded in sample buffer containing 5% 2-mercaptoethanol (reducing conditions). Electrophoresis was carried out at 150 V and terminated when the dye front reached the bottom of the gel. Proteins were transferred to a polyvinylidene fluoride membrane using a semi-dry transfer buffer (10% methanol; pH 9.0) and semi-dry transfer apparatus (Trans-Blot SD, SDS-PAGE cell; Bio-Rad Laboratories; Hercules, CA) at 250 mA for 45 min. After transfer, the membrane was blocked for 1 h with 5% skim milk made in TWEEN-20 Tris-base solution (TTBS) buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, at pH 7.5) at 22°C. The membrane was then incubated with primary antibodies overnight at 22°C. Antibodies included a polyclonal recombinant human stanniocalcin-1 (hSTC) antisera (21, 41) diluted 1:40,000 in TTBS, or β-actin antisera diluted to 1:10,000 in TTBS. The next day, the membrane was washed 3 times for 10 min with TTBS before being incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG for 45 min at 22°C (GE Healthcare; Baie d’Urfe, Canada), diluted 1:40,000 in TTBS. For detection, the membrane was washed 3 times for 10 min in TTBS then stained using an enhanced chemiluminescence system, a chemiluminescent, nonradioactive method to detect antibodies, according to the manufacturer’s instructions (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Blots were visualized by exposing the membranes to chemiluminescence-detecting film.

qRT-PCR. For mRNA analysis, samples from the SFO, cerebellar cortex, and kidney were immediately placed in TRIzol (Invitrogen, Carlsbad, CA) and homogenized using a motorized pestle homogenizer. Total RNA was then isolated according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Extracted RNA was resuspended in 30 μl of diethyl pyrocarbonate-treated water, and aliquots were stored at −80°C until use. Total RNA was assessed for purity (A260/A280) and quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA) (6, 57, 58).

Relative gene expression was determined using TaqMan STC-1 gene expression assay and TaqMan one-step RT-PCR master mix (Applied Biosystems, Foster City, CA). The expression of STC-1 was computed relative to the expression of the reference gene, GAPDH, using the comparative cycle threshold (Cₜ) method. Reactions were conducted using 15 μl of solution that contained 25 ng of total RNA with a 260/280 absorbance ratio of not less than 1.7. Each reaction was performed in quadruplicate on a clear 384-well plate that was sealed with an adhesive film prior to analysis. Reverse transcription was first carried out for 30 min at 48°C followed by an enzyme activation phase of 10 min at 95°C. The amplification reaction was run for 40 cycles alternating between 95°C and 60°C for 15 s and 1 min, respectively. All steps were performed on an ABI Prism 7900 HT sequence detector and used Sequence Detection Software 2.0 (Applied Biosystems, Foster City, CA) for analysis. Efficiencies above 90% were deemed acceptable.

STC-1 immunochemistry. Rats (n = 5) were deeply anesthetized with equithesin (0.3 ml/100 g body wt ip) and perfused transcardially with 200–300 ml 0.9% physiological saline, followed by 450 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and placed overnight in the fixative at 4°C. Brains were then gradually dehydrated through a series of alcohols and placed in xylene followed by paraflin wax (67). Serial, transverse sections (6 μm) through the region of SFO were cut on a microtome, mounted on double-gelatinized microscope slides, and placed on a slide warmer. Tissue sections were later deparaffinized in xylene and rehydrated using graded alcohol solutions. Sections were equilibrated using three 20-min washes of PBS (pH 7.4) and then underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate/0.05% Tween; pH 6.0) heated to 90–95°C in a microwave for 15 min (61). Slides were dehydrated, and endogenous peroxidase activity was inhibited by exposing the sections to a 1% H₂O₂ solution for 10 min. Sections were washed in PBS and allowed to incubate overnight at 22°C in primary polyclonal rabbit STC-1 antiserum (1:750 in PBS/0.3% Triton-X 100 and 4% normal goat serum) (59, 67). The STC-1 antiserum employed was generated in rabbits against hSTC (59, 67) and has previously been characterized for specificity (8, 21, 42). The sections were rinsed in PBS and exposed to goat anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA) for 1 h, followed by 75 min in ABC reagent (ABC Elite kit; Vector Laboratories). Following three rinses in PBS, the sections were immersed for 40 min in a solution of 0.05% 3,3’-diaminobenzidine containing 0.01% H₂O₂. The sections were rinsed and stored in PBS. Some tissue sections were dehydrated, stained with thionin, and cover-slipped for identification of cytoarchitectonic boundaries, whereas adjacent sections were only dehydrated and cover-slipped so as to not hinder the visualization of the reaction product.

Two types of controls for STC-1 immunoreactivity were used: omission of the primary antibody and the preabsorption of the antisera with an excess of human recombinant STC-1 antigen. Under these conditions, no STC-1 immunoreactivity was demonstrated.

Cells containing STC-1 immunoreactivity were visually identified on sections containing the SFO using bright-field microscopy (Letiz Diaplan). Digital images of these labeled cells were obtained with a Nikon DS-Fi1 camera and NIS Elements Basic Research 3.0 software (Nikon Canada, Mississauga, ON, Canada). The location and distri-
bution of STC-1 immunoreactive cells were mapped onto camera lucida projection drawings of the SFO. The atlas and nomenclature of Paxinos and Watson (43) were used for the identification of the central structures.

**Cannula implantation into the SFO.** Animals were anesthetized using equithesin (300 mg/kg ip; n = 12) and placed in a Kopf stereotaxic frame. A small midline incision was made in the skin overlying the cranium over the landmark bregma. A small 2-mm burr hole was drilled using stereotaxic coordinates (−0.8 mm rostrocaudal and −1.7 mm mediolateral to bregma, and 5 mm ventral to the cortical surface) (43) to target the SFO. A guide cannula made from a blunted 27-gauge stainless-steel needle was implanted at a 25° angle to the vertical meridian to avoid the sagittal sinus. The guide cannula was stereotaxically lowered to within 0.5 mm of the dorsal cortical surface of SFO using coordinates determined from the atlas of Paxinos and Watson (43). The guide cannula was then secured to the skull using jeweler’s screws and dental cement. The cement was allowed to dry, and the incision was sutured closed around the skull using 5-0 nylon. The animals were given postoperative care (3 ml ip of saline and an injection of the analgesic meloxicam) and allowed to recover for 1 wk following surgery before drinking experiments were conducted. Food and water were available ad libitum during the recovery period.

**Intra-SFO injections.** Injectors made from 33-gauge stainless-steel tubing cut to a length 0.5 mm longer than the guide cannula to ensure that the tip of the injector would end at or just below the dorsal surface of the SFO, were attached to polyethylene (PE)-10 tubing connected to a 1-μl Hamilton syringe. At 0700, injections (200 nl) of either the vehicle (0.9% saline), ANG II (250 ng/μl; Sigma Aldrich, St. Louis, MO), STC-1 (17.6 or 176 nM), a combination of vehicle (100 nl) followed by ANG II (100 nl), or a combination of STC-1 (100 nl; 17.6 or 176 nM) followed by ANG II (100 nl; 250 ng/μl) were made into SFO over a 30-s period. After an individual injection, the injector was allowed to remain in place for another 30 s. The injector was then removed and replaced with the obturator, and the animal was placed within a clear Plexiglas chamber (60 × 60 × 60 cm) that had a Richter tube with a nonspill spout attached containing 25 ml of tap water. Sham injections were also performed, during which time the injector was inserted and removed without making an injection. The order of injections or sham injection was randomized for each animal.

Each animal received injections of all compounds and sham injections. Only one compound or sham injection was made in each animal on one day. Different injections into the SFO were made on consecutive days. Measurements of cumulative water intake were made for each animal at 5, 10, 15, 30, and 60 min after the drug or sham injections into the SFO by direct reading from the Richter tube.

After all animals had been tested with all compounds or sham injection, the animals were deeply anesthetized and perfused transcardially with 50 ml of physiological saline followed by 50 ml of 10% buffered formalin. Brains were removed and stored in fixative 2–3 days prior to sectioning in a cryostat. Frozen, transverse 50-μm sections of the forebrain were cut through the region of the SFO, mounted on gelatinized slides, and stained with thionin for identification of cytoarchitectural boundaries to determine placement of the guide cannula by observing the cannula tract. The location of the injection sites was determined by extrapolation of the end of the cannula tract.

**Statistical analysis.** Values for water intake are presented as means ± SE. The qRT-PCR data were analyzed using an ANOVA for repeated measures. The amount of water drunk and time course of the water intake following the different injections was analyzed using a two-way ANOVA to determine differences between time and treatment, followed by a post hoc Bonferroni test when the ANOVA indicated statistical significance. A P < 0.05 was taken to indicate statistical significance.

**RESULTS**

**STC-1 protein and mRNA expression in SFO.** Western blot analysis of the punches of the SFO demonstrates the presence of STC-1 protein within SFO (Fig. 1). These levels were compared with those found within the cerebellum, as this region has previously been reported to exhibit STC-1 protein expression (6, 68, 69). SFO expressed considerably more STC-1 protein than that observed within the cerebellum (Fig. 1A).

The SFO samples analyzed using qRT-PCR indicate a relative gene expression of STC-1 mRNA that was approximately a 7.5-fold greater (P < 0.005) than that of the cerebellum. However, gene expression within SFO was considerably less (P < 0.01) than that expressed within the kidney (Fig. 1B).

**Distribution of STC-1 immunoreactivity in SFO.** Figure 2 shows an example of STC-1 immunoreactivity within the SFO, and Fig. 3 summarizes the distribution of cells within the SFO that contained the STC-1 immunoreactivity. STC-1-labeled cells were found throughout the rostrocaudal extent of the nucleus when examined in the transverse plane. However,

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Fig. 1. Western blot (A) showing the presence of STC-1 protein within SFO and cerebellum (Cere). Note that STC-1 is more highly expressed in SFO compared with the cerebellum. Note that the recombinant human stanniocalcin-1 (rhSTC-1) protein runs at a higher apparent molecular weight on SDS PAGE gels as also been reported by others (56) compared with the lighter molecular weight STC-1 in the tissue. B: STC-1 mRNA expression is detected by quantitative RT-PCR. The mRNA species is more highly expressed in SFO (P < 0.05) than cerebellum, but significantly (P < 0.05) less than that within the kidney. B: bars with different letters are significantly (P < 0.05) different from each other.
when viewed in the coronal plane, many of the labeled cells appeared to be mainly localized to the highly vascularized central area (Figs. 2 and 3). Additionally, many of the labeled cells in the SFO tended to be scattered in close proximity to large capillaries or to the ependymal wall. STC-1-like immunoreactivity was also observed within some of the cells of the choroid plexus in this region of the third ventricle (Fig. 2). SFO cells, and those of the choroid plexus both showed strong cytoplasmic immunoreactivity for STC-1. The immunoreactive STC-1 located within SFO cells was observed in a granular, punctate pattern (Fig. 2).

Effects of STC-1 in SFO on water intake. The effects of STC-1 injections at histologically verified sites within SFO (Fig. 4; n = 8) are summarized in Figs. 5 and 6. Animals receiving microinjection of both dosages of STC-1 into the SFO drank the same total amount of water over the 1-h recording period as control animals that received sham injections or injections of the vehicle (Fig. 5A). In addition, the time course of the drinking response in these STC-1-injected animals was not different compared with control animals that received sham injections or injections of the vehicle (Fig. 5A). However, at the 15-min time point after the injections of STC-1 into the SFO, there appeared to be a trend toward a reduction in water intake (176 nM; 0.69 ± 0.22 ml/100 g body wt; P < 0.055) compared with both sham (1.01 ± 0.22 ml/100 g body wt) and vehicle injections (0.94 ± 0.06 ml/100 g body wt) (Fig. 5B).

As expected (13, 25, 31), animals that received injections of ANG II into the SFO drank significantly (P < 0.01) more water beginning at 10 min after the injection compared with sham or vehicle-injected controls (Fig. 5A). STC-1 (17.6 or 176 nM) injections made immediately prior to the ANG II injection attenuated (P < 0.01) the water intake induced by ANG II starting at about 10 min and lasting throughout the 1-h period compared with ANG II alone or ANG II after vehicle injection (Figs. 5B and 6). There were no differences in the ANG II drinking responses between the two amounts of STC-1 injected. The attenuation of the drinking response to ANG II occurred between 15 and 60 min after the STC-1 injections. Over the 1-h period, control animals that received STC-1 in combination with ANG II drank significantly (P < 0.05) less water (1.30 ± 0.46 ml/100 g body wt) compared with vehicle + ANG II-injected animals (2.36 ± 0.39 ml/100 g body wt; Fig. 6).

Sham-injected animals did not alter the drinking of water compared with control-injected or STC-1-injected animals.
In addition, in four animals in which the injection cannula missed the SFO and was histologically verified within the dorsal medial septum, STC-1 (176 nM; 1.2 nM/11006 0.3 ml/100 g body wt), vehicle (1.5 nM/11006 0.3 ml/100 g body wt), or ANG II (1.4 nM/11006 0.2 ml/100 g body wt), failed to alter the amount of water intake compared with sham-injected animals.

DISCUSSION

This study has provided not only the first evidence that STC-1 is produced in a circumventricular organ but also that it is involved in body fluid homeostasis. The SFO was found to contain neurons that expressed STC-1 immunoreactivity and contained both protein and gene expression for STC-1. The findings that STC-1 is produced within the SFO combined with the previous finding that SFO contains putative STC-1 receptors (45), suggests that STC-1 plays an important role in the function of neuronal systems controlling body fluid balance (7, 15, 36, 37). In addition, as SFO neurons have been shown to contain STC-1 binding activity (45) and STC-1 is constitutively expressed within the SFO, this suggests that STC-1 may also function in an autocrine/paracrine manner (24, 34) in SFO neuronal function (6, 68, 69). However, the possibility also exists that STC-1 acts as a neurotransmitter within the SFO.

The intake of fluid by mammals is an essential physiological response to signals of dehydration (13, 25). These signals are thought to exert their effects through the activation of osmo-sensitive neurons in response to a loss of intracellular fluid, or ANG II-sensitive neurons in response to extracellular dehydration (13, 33, 60). This osmotic change and ANG II cause an activation of mechanosensitive cation channels within the osmoreceptor and/or neuron and the resulting depolarization of the neuron is mediated by increases in intracellular calcium concentration (39, 40). Neurons sensitive to osmolality changes and circulating ANG II are thought to exist in a number of central structures, although most are found within the laminae terminals of the forebrain. Of these structures, the

Fig. 4. Brightfield photomicrograph (A) and transverse section through the region of the SFO (B) showing the location of injections sites (circles). A: an asterisk (*) shows the guide cannula tract, while the dashed-dotted lines show the outline of necrotic tissue due to the injector cannula placement that extended out of the guide cannula towards the dorsal surface of the SFO. Refer to Fig. 2 for a list of abbreviations. B: calibration mark represents 250 μm.

Fig. 5. The effects of STC-1 (17.6 nM; n = 7 and 176 nM; n = 5) and ANG II (250 ng/µl; n = 7), combination of STC-1 and ANG II (n = 7) on cumulative water intake over a 1-h time period after injection into SFO. A: effect of vehicle or sham injection, ANG II, and the two different doses of STC-1 on water intake. Note that injections of the vehicle into SFO did not alter the drinking response compared with sham injections (A). Additionally, note that both doses of STC-1 had similar effects on drinking that were significantly less (*P < 0.01 and †P < 0.05) than those observed with ANG II alone (A). *: the asterisks (*) indicate statistical significance (P < 0.01) between ANG II response and all other compounds tested. †: statistical differences (P < 0.05) between ANG II and vehicle are indicated.
SFO is known to have an important role in fluid balance, as its fenestrated capillaries allows this brain region to easily respond to circulating signals, such as osmoles and hormones (12, 15). One of the hormones most important in signaling fluid homeostasis at the SFO is ANG II involved in signaling extracellular dehydration and volume challenges. Administration of ANG II into the SFO has previously been shown to elicit a drinking response (31, 53, 54).

The finding of STC-1 mRNA and protein expression within the SFO is a novel observation as the cerebellum and more recently, the nucleus of the solitary tract (6) are the only other extracortical central sites that have been shown to contain STC-1 protein (69). STC-1 protein and mRNA were more highly expressed within the SFO compared with the cerebellum. It may be argued that this difference may have resulted from nonspecific sampling of the tissues. However, this is unlikely, as the punches taken from each area were of the same size and taken from the same regions in all animals. In addition, protein and RNA samples were standardized to contain the same amount of protein and RNA in each. Although it is possible that there could be degradation of STC-1 protein or RNA in any given sample, all samples from both locations were processed similarly. As well, the expression profile of STC-1 protein and mRNA was consistent in all animals used. The use of the housekeeping gene GAPDH during qRT-PCR procedures and using the comparative Ct method ensured equal loading and amplification. Recently, this housekeeping gene has been used to reliably identify changes in STC-1 gene regulation in samples obtained from the rat kidney (57, 58). Thus, it can be concluded that the differences seen between these two central areas are due to the amount of available STC-1 protein or mRNA.

STC-1 protein and gene expression in the SFO are consistent with the immunohistochemical data showing that cells expressing STC-1 immunoreactivity are found throughout the SFO. Of interest is that STC-1-labeled cells are found predominantly within regions of the SFO known to have a high capillary density, perivascular spaces and to contain capillaries with endothelial fenestrations (19). Thus, these regions of the SFO are highly suited for chemoreception of blood-borne substances (7, 12, 15, 36, 37). These highly vascularized regions are also known to contain neurons responsive to circulating ANG II (20). Excitation of these neurons by ANG II has been shown to activate neuronal circuits that lead to increased drinking behavior (13, 36, 37). Thus, it was not unexpected to find that administration of STC-1 into the SFO affected ANG II-induced water intake, especially as it has previously been shown that the same general region of the SFO contains STC-1 binding sites (45). The finding that STC-1 binding sites are localized to the same general areas of the SFO, in which cells that express STC-1 are found. This suggests that STC-1 in SFO, as previously indicated for other tissues, may act in an autocrine/paracrine manner (24, 63), in addition to possibly responding to blood-borne STC-1.

Administration of STC-1 directly into SFO did not alter normal water intake compared with control injections. However, when these animals were challenged with an injection of ANG II into SFO, STC-1 attenuated the drinking response induced by ANG II. Although the mechanism by which this inhibition of ANG II-induced water intake by STC-1 occurs is not known, ANG II has been shown to increase drinking behavior through a neuronal calcium-dependent process involving L-type calcium channels. In myocardial cells, STC-1 has been reported to block these calcium channels (51). Additionally, STC-1 has been shown to increase mitochondrial uptake of calcium (10), a mechanism that has also been suggested to occur in central neurons (68–70). Thus, it is not unreasonable to suggest that STC-1 reduces the excitability of SFO neurons to ANG II by mitochondrial buffering of available intracellular calcium.

Injections of ANG II elicited a drinking response in all animals that had injector placement within the SFO. Animals in which the injector was located outside the SFO region not only failed to respond with drinking to ANG II microinjection, but also to microinjections of STC-1. These observations are of some significance as they suggest that the effects of STC-1 on drinking when microinjected into the SFO were not due to nonspecific actions of either ANG II or STC-1. Consistent with this suggestion are the findings that AT1Rs are found throughout the laminae terminalis, including SFO (5, 38), as are STC-1 receptors (45).

Perspectives and Significance

The data obtained in this study suggest that STC-1 within this forebrain circumventricular organ functions in the control of fluid intake. It is interesting to note that hypertonicity and increased osmolality induce STC-1 in different tissues (52, 57, 58). Changes in plasma osmolality and hypertonicity, like ANG II, increase intracellular cytosolic calcium levels within SFO neurons (17, 27, 28). Chronic elevated levels of plasma osmolality, hypertonicity, or ANG II not only stimulate SFO neurons, but also increase discharge by these neurons. This increased discharge is likely due to elevated levels of intracellular cytosolic calcium. It is well known that activity-dependent intracellular calcium overload may result in neuronal apoptosis (4, 16, 23). Thus, the possibility exists that one of the main functions of STC-1 within SFO is to reduce the influx and the rise in intracellular calcium levels (10, 70). Therefore, STC-1 may be involved in maintaining the integrity of SFO.
neurons by functioning as an antiapoptotic agent, as suggested for cortical neurons (70), kidney collecting ducts (9), and cardiomyocytes (30). Furthermore, Sazonova et al. (49) have reported that STC-1 receptors within the kidney are not downregulated in the presence of elevated STC-1, indicating that these cells continue to regulate cytosolic calcium levels despite chronic stimuli that would further induce elevated intracellular calcium. Such a cellular mechanism would be of considerable importance in SFO, as it is not only constantly exposed to changes in circulating ANG II, but must be able to respond appropriately to activate neuronal systems to restore body fluid and electrolyte balance. Finally, STC-1 may also function in the SFO to terminate ANG II-stimulated drinking by increasing intracellular uptake of Ca2+ by mitochondria (10).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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