Original Article

Activity of Protein Kinase C-α Within the Subfornical Organ Is Necessary for Fluid Intake in Response to Brain Angiotensin

Jeffrey P. Coble, Ralph F. Johnson, Martin D. Cassell, Alan Kim Johnson, Justin L. Grobe, Curt D. Sigmund

Abstract—Angiotensin-II production in the subfornical organ acting through angiotensin-II type-1 receptors is necessary for polydipsia, resulting from elevated renin–angiotensin system activity. Protein kinase C and mitogen-activated protein kinase pathways have been shown to mediate effects of angiotensin-II in the brain. We investigated mechanisms that mediate brain angiotensin-II–induced polydipsia. We used double-transgenic sRA mice, consisting of human renin controlled by the neuron-specific synapsin promoter crossed with human angiotensinogen controlled by its endogenous promoter, which results in brain-specific overexpression of angiotensin-II, particularly in the subfornical organ. We also used the deoxycorticosterone acetate-salt model of hypertension, which exhibits polydipsia. Inhibition of protein kinase C, but not extracellular signal–regulated kinases, protein kinase A, or vasopressin V₁A and V₂ receptors, corrected the elevated water intake of sRA mice. Using an isoform selective inhibitor and an adenovirus expressing dominant negative protein kinase C-α revealed that protein kinase C-α in the subfornical organ was necessary to mediate elevated fluid and sodium intake in sRA mice. Inhibition of protein kinase C activity also attenuated polydipsia in the deoxycorticosterone acetate-salt model. We provide evidence that inducing protein kinase C activity centrally is sufficient to induce water intake in water-replete wild-type mice, and that cell surface localization of protein kinase C-α can be induced in cultured cells from the subfornical organ. These experimental findings demonstrate a role for central protein kinase C activity in fluid balance, and further mechanistically demonstrate the importance of protein kinase C-α signaling in the subfornical organ in fluid intake stimulated by angiotensin-II in the brain. (Hypertension. 2014;64:00-00.) • Online Data Supplement

Key Words: adenoviridae • angiotensin-II • brain • drinking • protein kinases

Angiotensin-II (Ang-II) is a known dipsogen, which induces drinking in rats when administered peripherally or centrally.1-3 Mice fail to drink water in response to peripheral Ang-II but do so after Ang-II is injected into the brain.3 Central Ang-II–induced fluid intake has been shown to be mediated by an Ang-II type-1 receptor (AT₁R)–dependent pathway.4-6 AT₁R, a G-protein–coupled receptor, which on activation by Ang-II induces release of Gₛₐ₁, that can activate protein kinase C (PKC) via diacylglycerol and inositol triphosphate–induced calcium release.7 AT₁R can also activate extracellular signal–regulated kinases 1 and 2 (ERK1/2) independently from the PKC pathway.10 PKC and ERK1/2 have both been shown to affect central Ang-II–mediated fluid intake and blood pressure, but their effects depend on their site of action in the brain.11-13

Ang-II injected into the lateral ventricle or directly into the subfornical organ (SFO) induces water intake within minutes through AT₁R.2 Neuronal excitability is increased on administration of Ang-II centrally or directly into the SFO.14,15 SFO neurons express outwardly rectifying potassium channels (Iₒᵣ)16,17 and the excitatory effects of Ang-II were reported to be mediated by AT₁R-dependent inhibition of Iₒᵣ.17,18 Ang-II activation of AT₁R increases the activity of PKC-α centrally, and PKC-α mediates the AT₁R-dependent inhibition of Iₒᵣ.19,20 Central inhibition of the conventional PKC isoforms, PKC-α/βII, with Go-6976 attenuates the induction of water intake in rats caused by the administration of Ang-II within the lateral ventricle.20

Double-transgenic (sRA) mice overexpressing human renin and angiotensinogen selectively in the brain exhibit both hypertension and a marked increase in fluid intake.21 Normal C57BL/6 mice treated with deoxycorticosterone acetate and high salt (DOCA-salt) also exhibit polydipsia.22 It is well recognized that the DOCA-salt model of hypertension is a model of elevated brain Ang-II activity. Indeed, we recently showed that many of the cardinal phenotypes observed in the sRA transgenic model (hypertension, polydipsia, and elevated resting
metabolic rate) are also observed in DOCA-salt mice. The human angiotensinogen transgene is robustly expressed in the SFO in sRA mice, and Cre-recombinase-loxP-mediated ablation of its expression selectively in the SFO blunts fluid intake. Selective deletion of AT₁R in the SFO of DOCA-salt–treated mice carrying a conditional allele of AT₁R (AT₁R<sup>fl</sup>) similarly blunts fluid intake. These studies highlight the importance of both Ang-II production and AT₁R action in the SFO as an important mediator of the polydipsic response. Herein, we tested the hypothesis that PKC-α is an important mediator of Ang-II–dependent fluid intake in sRA and DOCA-salt mice, and that activation of PKC independent of Ang-II is sufficient to stimulate fluid intake rapidly.

**Methods**

Specific protocols, drug administration, and detailed methods are provided in the expanded Methods in the online-only Data Supplement.

**Animals and Surgery**

Male and female transgenic mice 12 to 20 weeks were used in this study and were Institutional Animal Care and Use Committee approved in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. sRA mice are double-transgenic mice derived from a cross of mice expressing human renin under the neuron-specific synapsin promoter (sR) with mice expressing human angiotensinogen under the control of its own endogenous promoter (A). Littermates were used as controls. For acute intracerebroventricular injection or placement of a chronic intracerebroventricular cannula, the lateral ventricle was targeted and injected as described previously.22 Intracerebroventricular minipumps (Alzet, Illinois, Chicago, IL) were inserted at the start of the second week of DOCA-salt.

**Statistics**

We analyzed the data with 1- or 2-way ANOVA, with repeated measurements as appropriate. Bonferroni multiple comparisons procedures were used to explore treatment effects further. If equal variance or normality failed, we used nonparametric analysis of our data, such as Mann–Whitney U or Wilcoxon tests. We considered significant differences at P<0.05, and all data plotted are mean±SEM.

**Results**

### Intracerebroventricular Drug Panel in sRA Mice

As a first pass to screen mechanisms mediating brain Ang-II–induced polydipsia, a panel of drugs was individually injected intracerebroventriculatly into sRA mice, and water intake after each administration was measured (Figure 1A). Sufficient time was allowed for recovery after each drug, and this was validated by measuring water intake after intracerebroventricular injection of artificial cerebrospinal fluid (aCSF) the day before the next drug was tested. Consistent with our previous study, water intake was elevated in sRA mice when compared with controls (Figure 1B). The increase in water intake in sRA mice was corrected by bisindolylmaleimide (BIM), a nonselective PKC inhibitor, which acts by blocking the activation domain (genotype×BIM interaction, P=0.01; Figure 1B). FR180204, a direct inhibitor of ERK1/2, significantly decreased water intake of both littermate control and sRA mice; however, there was no significant interaction between genotype and drug (P=0.63). Water intake of sRA mice was still elevated when compared with littermate controls after FR180204 (P<0.01). Neither conivaptan, an antagonist of arginine vasopressin V₁A and V₂ receptors, nor H-89, an inhibitor of PKA, had any effect on water intake in sRA and controls. Consistent with the actions of BIM, inhibiting the regulatory domain of PKC with calphostin C corrected the elevated water intake in sRA mice such that it was not significantly different from controls (P=0.83). Polydipsia in sRA mice was retained at the end of the experiment although it was blunted. These data suggest that the multiple testing procedure was not deleterious, and the washout periods were sufficient.
sufficient. Thus, this initial screen identified the PKC pathway as an important mechanism regulating brain Ang-II–induced polydipsia in sRA mice.

Conventional PKCs Are Necessary to Elevate Water Intake Because of the Brain Renin–Angiotensin System

A dose–response study was performed to verify that central activation of PKC mediates brain Ang-II–induced polydipsia. BIM had no significant effect at a dose of 32 pg. Starting at 3.2 ng, BIM significantly decreased 24-hour water intake in sRA when compared with control mice (Figure 2A). There was a significantly greater maximum change in water intake from vehicle in sRA when compared with control mice (Figure 2B). Starting at 1 ng, Gö-6976, a selective PKC-α/βII inhibitor, significantly decreased water intake in sRA mice (Figure 2C), and the maximum change in water intake because of Gö-6976 was significantly greater in sRA than in littermate controls (Figure 2D). Thus, conventional isoforms of PKC mediate brain Ang-II–mediated polydipsia in this model.

PKC-α From the SFO Mediates Brain Renin–Angiotensin System Polydipsia

We and others have demonstrated that an intracerebroventricular injection of adeno virus targets the SFO.17,27–29 To confirm this in the current cohort, adeno virus encoding Cre-recombinase was first injected intracerebroventricular into a reporter model that specifically detects Cre-mediated recombination (ROSA-CAG-LSL-tetTomato mice). One month after intracerebroventricular adeno virus encoding Cre-recombinase, recombination was only found within the SFO (Figure 3B) and tanyocytes of the central canal (Figure 3F). Nuclei important for cardiovascular control, such as the median preoptic nucleus (Figure 3A), organum vasculosum of the lamina terminalis, supraoptic nucleus (Figure 3C), paraventricular nucleus (Figure 3D), rostral ventrolateral medulla (Figure 3E), area postrema, and nuclei tractus solitarius (Figure 3F), were largely devoid of evidence of recombination. Thus, intracerebroventricular adeno virus selectively infects cells within the SFO.

An adeno virus (Ad)-DN-PKC-α was used to determine whether PKC-α activation mediates brain Ang-II–induced polydipsia and to determine whether this occurs within the SFO. In this experiment, we provided sRA mice with a 2-bottle choice of deionized water and isotonic saline. Nine to 10 days after intracerebroventricular Ad-DN-PKC-α, the intakes of water (Figure 4A and 4B), 0.15 mol/L NaCl (Figure 4C and 4D), total fluid (Figure 4E and 4F), and total sodium (Figure S1A in the online-only Data Supplement) of sRA mice were attenuated, whereas control mice were unaffected. There was no difference between the genotypes in salt preference (Figure S1B). It is important to note that the decrease in fluid intake after SFO-selective Ad-DN-PKC-α was approximately half that observed after intracerebroventricular BIM or Gö-6976. An 11% decrease in food intake (Figure S1C and S1E) contributed to an attenuation of total sodium consumption in sRA mice; however, there was no change in body weight (Figure S1D). These data suggest that the increase in fluid and sodium intake in response to increased brain Ang-II requires PKC-α activity within the SFO.

PKC Activity Is Necessary for DOCA-Salt Polydipsia

We next tested the role of central PKC in an independent model of hypertension. Intracerebroventricular osmotic mini-pumps containing aCSF or BIM were placed into DOCA- or sham-treated mice at the start of the second week after initiation of DOCA-salt treatment. Chronic intracerebroventricular BIM blunted the increase in fluid intake caused by DOCA-salt (Figure 5A). DOCA-salt mice treated with aCSF drank more water and 0.15 mol/L NaCl than sham mice and consumed more sodium (Figure 5B and 5D), effects blunted by BIM treatment. DOCA-salt mice did not exhibit a significantly different preference for saline or food intake when compared with littermate controls, and BIM had no effect on these measurements (Figure 5E and 5F). Thus, as observed in sRA mice, central PKC also plays a role in fluid intake caused by DOCA-salt.

Central PKC Activity Is Sufficient to Increase Water Intake

Given the potential role of PKC-α, we next determined whether PKC-α becomes activated in primary cells cultured from the SFO.17,27 Cells were treated with vehicle or phorbol-12-myristate-13-acetate (PMA) for 15 minutes and phosphorylated PKC-α was visualized by immunofluorescence. Approximately 12% of the cells exhibited a positive signal.

**Figure 2.** Drinking response to protein kinase C blockade in sRA mice. Change in 24-hour water intake of sRA and littermate control mice because of intracerebroventricular (ICV) aCSF (artificial cerebrospinal fluid) to increasing doses of bisindolylmaleimide (BIM) (A; sRA n=7 and control n=9) or Gö-6976 (C; sRA n=6 and control n=6). There are significant interactions between genotype and dose of BIM and Gö-6976. The maximum response to a change in water intake because of ICV BIM (B) or Gö-6976 (D) is shown. *P<0.01.
PKC-α was observed as punctate staining throughout the cytoplasm in vehicle-treated cells (Figure 6A and 6B), whereas in PMA-treated cells, the PKC-α signal was localized primarily at the plasma membrane (Figure 6C and 6D).

Finally, we tested whether a direct pharmacological activator of conventional and novel PKCs (PMA) when injected into the lateral ventricle of water-replete C57BL/6 mice would be sufficient to induce water intake. PMA induction of water intake occurred after 5 minutes, and most of the cumulative water intake occurred within 15 minutes of injection (Figure 7A). The PMA-induced water intake during a 5-hour period, as measured by the area under the curve, was markedly elevated when compared with aCSF (Figure 7B). Whereas when PMA induced water intake acutely, it did not affect 24-hour total consumption of water when compared with aCSF, suggesting that the agent is rapidly cleared (aCSF, 3.02±0.16 mL/d versus PMA, 2.08±0.58 mL/d; P=0.195). Blocking central PKC activity by pretreating mice with intracerebroventricular BIM almost completely blunted the PMA induction of water intake ≥30 minutes (Figure 7C and 7D). Thus, central PKC activity is sufficient to induce water intake in water-replete mice.

Discussion

There are several findings from this study. First, the activity of central PKC, but not of ERK or PKA, is required to mediate polydipsia in sRA mice. Second, the action of central PKC in mediating drinking in sRA mice is through the PKC-α/βII isoform. Third, blocking the activity of PKC-α selectively in the SFO blunts, but does not fully abolish, increased water and sodium intake in sRA mice. Fourth, as observed in sRA mice, inhibition of central PKC activity blunts increased water intake in DOCA-salt mice. Finally, inducing PKC activity with PMA caused an increase in cell surface localization of phosphorylated PKC-α in cultured cells from the rat SFO, and central PKC activity is sufficient to increase water intake in water-replete mice. Together, these findings highlight an important role for central PKC-α as a regulator of fluid homeostasis in 2 different models that exhibit increased brain renin–angiotensin system activity and suggest that PKC-α activity specifically in the SFO plays a significant role in this response.

Both sRA and DOCA-salt models exhibit increased brain angiotensin system activity, and we showed that the drinking responses in both models were blocked by AT1R antagonist losartan.7,22,23 We recognize that sRA is an overexpression model that serves as a genetic tool enabling the demonstration of what occurs in response to endogenous Ang-II formation within the central nervous system. That both sRA and DOCA-salt mice exhibit similar phenotypes, and DOCA-salt is recognized as a model that has a central Ang-II component validates their use herein.21,22 Moreover, we demonstrated that ablation

Figure 4. Drinking response to dominant negative protein kinase C (DN-PKC)-α. Intakes of water (A and B), 0.15 mol/L NaCl (C and D), and total fluid (E and F) were attenuated by intracerebroventricular adenovirus (Ad)-DN-PKC-α from baseline (BL) in sRA (gray; n=13) but not in littermate control mice (black; n=14). There are statistically significant interactions between genotype and virus for intakes of water, 0.15 mol/L NaCl, and total fluid. *P<0.05, genotype×virus interaction.
of Ang-II overproduction selectively in the SFO attenuates polydipsia in sRA transgenic mice, and selective ablation of endogenous AT₁_R in the SFO attenuates DOCA-salt–induced polydipsia. Therefore, these findings demonstrate that both Ang-II production and AT₁_R action in the SFO are mechanistically necessary for polydipsia induced by the brain renin–angiotensin system. The importance of the SFO for water and saline intake has been shown by lesion studies and by measurements of c-fos expression as an indicator of neuronal activity in the SFO in response to various dipsogenic stimuli.

Herein, we show that targeting PKC-α in the SFO through an intracerebroventricular injection of a virus expressing DN-PKC-α attenuates the elevated fluid and sodium intake in sRA mice. These data extend our previous finding to suggest that PKC-α activity downstream of AT₁_R signaling in the SFO partially mediates Ang-II–induced polydipsia. When compared with the nonselective inhibitor of PKC, BIM, and the selective PKC-α/βII inhibitor, Gö-6976, SFO-selective Ad-DN-PKC-α was less effective in blunting fluid intake in sRA mice. First, through intracerebroventricular administration, the pharmacological inhibitors likely gain access to many nuclei in the brain and the effects on fluid intake are the sum of PKC inhibition at all of these sites. This would suggest that PKC-α signaling in the SFO regulates a portion of the total dipsogenic response. Second, Ad-DN-PKC-α infection of the SFO may have been incomplete, and as previously shown, adenoviral infection targets both glia and neurons in the SFO. Whereas our studies using intracerebroventricular adenovirus encoding Cre-recombinase in Cre-reporter mice indicates effective infection of the SFO, the number of

Figure 5. Role of protein kinase C (PKC) in mediating the drinking response to deoxycorticosterone acetate (DOCA)-salt. Total fluid (A), water (B), saline (C), total sodium (D), sodium preference (E), and food (F) intakes in sham (artificial cerebrospinal fluid [aCSF], n=5; bisindolylmaleimide [BIM], n=5) and 3-week DOCA-salt mice (aCSF, n=10; BIM, n=12) with chronic intracerebroventricular (ICV) aCSF (Alzet) or BIM (100 μg/d in aCSF). Preference for 0.15 mol/L NaCl in sham and in DOCA-salt mice with either chronic ICV aCSF or BIM (D). *P<0.05 Bonferroni post hoc comparison between genotypes or treatments.

Figure 6. Protein kinase C (PKC)-α activity in cultured cells from subfornical organ (SFO). Immunofluorescent images detecting nuclei (blue nuclei stained with DAPI [4',6-diamidino-2-phenylindole]) and phosphorylated-PKC-α in primary cells cultured from the SFO of newborn rats. Cells were treated with vehicle (A, B) or PMA (phorbol-12-myristate-13-acetate) (C, D) for 15 minutes. Scale bar, 100 μm.
In hypothalamic and brain stem calmodulin-dependent kinase II (CAMKII) because Gö-6976 corrected if both are inhibited. Furthermore, Ang-II increases activation of ERK1/2 in human adenocarcinoma cells. Finally, it is possible that some of the effects of pharmacologically inhibiting PKC are mediated by off-target inhibition of calmodulin-dependent kinase II (CAMKII) because Gö-6976 (a derivative from BIM) decreases the activity of CAMKII-α and CAMKII-β in vitro. In hypothalamic and brain stem cultures, the excitatory effects of Ang-II through AT 1R are attenuated with inhibition of either PKC or CAMKII but are corrected if both are inhibited. Third, other PKC isoforms that are effectively inhibited by BIM, but not by Gö-6976 or Ad-DN-PKC-α, may also play a role in mediating the Ang-II response. For example, Ang-II stimulation of human gloma cells activates PKC-ε and PKC-α. Inhibition of PKC-ε blocks Ang-II-inhibition of Na/K-ATPase in cardiomyocytes and Ang-II-induced activation of ERK1/2 in human adenocarcinoma cells.

Finally, it is possible that some of the effects of pharmacologically inhibiting PKC are mediated by off-target inhibition of calmodulin-dependent kinase II (CAMKII) because Gö-6976 (a derivative from BIM) decreases the activity of CAMKII-α and CAMKII-β in vitro. In hypothalamic and brain stem cultures, the excitatory effects of Ang-II through AT 1R are attenuated with inhibition of either PKC or CAMKII but are corrected if both are inhibited. Furthermore, Ang-II increases the expression of CAMKII-α in cultures of rat septum and hypothalamus. Inhibition of CAMKII via KN-93 attenuates central Ang-II induction of water intake. Attenuation of water intake via inhibition of CAMKII and PKC is not additive. CAMKII and PKC seem to mediate Ang-II induction of water intake specifically because their inhibition did not attenuate carbachol-induced water intake.

It is notable that when the 2-bottle choice paradigm was used for the Ad-DN-PKC-α studies, we observed a larger effect on saline rather than water intake in sRA mice. Previous studies implied that central PKC-α does not mediate intake of saline because of central Ang-II, which seems to contradict our results. However, in the previous studies, saline was not given as a choice with water, saline intake at a nonaversive concentration was not evaluated, and specifically PKC-α in the SFO was not inhibited. Interestingly, BIM blunted total fluid intake and saline intake in DOCA-salt mice, and although the decrease in water intake was not significant, there was no significant difference between water intake in DOCA-salt and sham mice after BIM. Thus, we cannot rule out that the effects of specifically inhibiting PKC-α in the SFO may differ from the effects of blocking all PKC isoforms throughout the brain. Also, the mechanisms of fluid intake in DOCA-salt mice are likely a sum of the effects of increased local Ang-II signaling in the brain, the stimulation of central mineralocorticoid receptors, and possibly an interaction between both. Indeed, evidence suggests that central mineralocorticoid receptors can sensitize rats to the pressor effects of Ang-II. It is unclear whether pressor responses to Ang-II can be extended to the dipsogenic responses although it is interesting to note that aldosterone increases calcium signaling in cultured SFO neurons in response to Ang-II.

In the initial drug screen, we observed that direct inhibition of ERK1/2 with FR180204 attenuated water intake in both sRA and littermate controls. The expression of ERK1/2 in the SFO increases in fluid-restricted mice. Daniels et al have shown that inhibition of ERK1/2 attenuates the acute increase in sodium appetite but not water intake of rats injected with Ang-II into the lateral ventricle. Thus, ERK1/2 may affect fluid intake by increasing the intake of a normally aversive concentration of saline. Because sodium appetite is not elevated in sRA mice and we measured only the intake of water in the initial screening experiment, the lack of a robust effect of FR180204 on water intake by sRA mice was not entirely unexpected.

We previously showed that hypertension in sRA mice is attenuated by a chronic, peripheral infusion of conivaptan or tolvaptan, but in the current study, we did not observe an effect on water intake. This suggests that central V 1A and V 1R (expressed in the hypothalamus, hippocampus, cortex, and cerebellum; GenSat.org) do not mediate the water intake because of elevated brain Ang-II. Although the V 1A and V 1R is not inhibited with conivaptan, it is unlikely that they mediate the polydipsia of sRA mice. Indeed, we previously showed that adrenal steroids mediate polydipsia in sRA mice because adrenalectomy completely blocks polydipsia.
Although previous studies and our present data demonstrate the necessity of central PKC, specifically PKC-α, for central Ang-II induction of water intake, we also show that activity of conventional or novel isoforms of PKC in the brain is sufficient to induce the intake of water in water-replete mice.\textsuperscript{11,20} This may occur as a result of PKC-α activation within the SFO. This conclusion is based on the observations that the induction of water intake in response to PMA follows a similar time-course to a central injection of Ang-II,\textsuperscript{2} that lesion of the SFO attenuates central Ang-II–induced polydipsia,\textsuperscript{40} our data show that PKC-α in the SFO is necessary to mediate the full extent of polydipsia in sRA mice, and our data showing cell surface–associated phosphorylated PKC-α in cultured cells from the rat SFO. We conclude that local production and action of Ang-II within the SFO increase PKC-α activity, which is necessary and sufficient for the elevated intake of water and nonaversive saline.

**Perspectives**

Polydipsia occurs in, and can aggravate, type 2 diabetes mellitus, heart failure, chronic kidney disease, chronic psychosis, and adverse reactions to drugs. We show that polydipsia caused by hyperactivity of brain angiotensin activity occurs through the activity of PKC-α within the SFO. We also show that central PKC activity (presumably PKC-α) is sufficient to induce water intake in water-replete mice. Understanding the molecular mechanisms of fluid intake will allow us to treat polydipsia pharmacologically.

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**Disclosures**

None.

**References**


### What Is New?
- Central activity of conventional or novel protein kinase C (PKC) is sufficient to induce water intake in water-replete mice.
- Fluid intake of both water and saline (0.15 mol/L NaCl) because of hyperactivity of the brain renin–angiotensin system is mediated through PKC-α within the subfornical organ.
- Central extracellular signal-regulated kinases 1 and 2, PKA, or vasopressin receptors V₁A and V₂R do not seem to mediate elevated water intake because of hyperactivity of the brain angiotensin system.

### What Is Relevant?
- Central PKC is both necessary and sufficient to induce water intake.
- Polydipsia resulting from increased activity of the brain angiotensin system is mediated through PKC-α within the subfornical organ.

### Novelty and Significance

**Summary**

We show that hyperactivity of the brain renin–angiotensin system increases intake of both water and saline. PKC-α activity within the subfornical organ is necessary for this polydipsia, and central extracellular signal-regulated kinases 1 and 2, PKA, and vasopressin receptors V₁A and V₂R do not seem to mediate polydipsia because of hyperactivity of the brain renin–angiotensin system. Furthermore, induction of central PKC is sufficient on its own to induce water intake.

**What Is New?**
- Central activity of conventional or novel protein kinase C (PKC) is sufficient to induce water intake in water-replete mice.
- Fluid intake of both water and saline (0.15 mol/L NaCl) because of hyperactivity of the brain renin–angiotensin system is mediated through PKC-α within the subfornical organ.
- Central extracellular signal-regulated kinases 1 and 2, PKA, or vasopressin receptors V₁A and V₂R do not seem to mediate elevated water intake because of hyperactivity of the brain angiotensin system.
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DATA SUPPLEMENT

Activity of PKC-α within the Subfornical Organ is Necessary for Fluid Intake due to Brain Angiotensin

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Supplemental Methods

Experimental mice: sRA mice (line 11110/2 x 4284/1) are double transgenic mice derived from a cross of mice expressing human renin under the neuron-specific synapsin promoter (sR) with mice expressing human AGT under the control of its own endogenous promoter (A). They were generated as described previously, and littermates were used as controls.\textsuperscript{1,2} C57BL/6 mice (stock 000664) were used for DOCA-salt and phorbol-12-myristate-13-acetate (PMA) studies. ROSA-CAG-LSL-tdTomato (stock 007914) mice were obtained from the Jackson Laboratory. We measured fluid balance in mice by either placing them in their home or a metabolic cage as described previously.\textsuperscript{2}

Pharmacology: aCSF (Alzet Inc.), BIM (32 ng in 2 μL DMSO; Cell Signaling), H-89 (4 μg in 2 μL DMSO; Cell Signaling), FR180204 (4 μg in 2 μL DMSO; Santa Cruz), conivaptan (0.8 μg in 2 μL aCSF; Baxter Healthcare), and calphostin C (0.4 μL in 2 μL DMSO; Sigma). Gö-6976 (Millipore), and PMA (Cayman Chemical) were injected as described previously.\textsuperscript{3}

ICV Cannula Placement: Specific cohorts of mice had cannulas (PlasticsOne, Inc.) placed into the lateral ventricle after which they recovered individually housed in a home cage on a hot pad with food and water. They were monitored for 24-hours. After 24-hours, the mice were returned to the vivarium. The mice were given an additional 7-day period in the vivarium to recover from the surgery before studies were performed. If it was evident that a mouse had ill effects of the surgery, it was killed before the study was started. The success rate for ICV cannula placement and injection was over 90%.

Acute ICV Injection: Select cohorts of mice were given isoflurane to induce (5% isoflurane in O\textsubscript{2}) and maintain (2% isoflurane in O\textsubscript{2}) anesthesia. The lateral ventricle was located via stereotaxic coordinates, and 1 μL of adenovirus (AdCre or Ad5CMV-DN-PKC-α, 1x10\textsuperscript{7} pfu/μL; University of Iowa Gene Transfer and Vector Core) was injected via a 1 μL Hamilton syringe over 5 min. The Hamilton syringe was then kept in place for an additional min before retracting it and suturing together the skin covering the cranium. Mice were then place in a home cage on a warm pad with food and water, and their recovery was monitored for 24 hours. After 24 hours, the mice were returned to a specific room in the vivarium dedicated for adenovirus. After 7 days, the mice were transferred and individually housed in a clean cage.

Specific Protocols by Figure.

Figure 1: ICV drug panel in sRA and littermate control mice.
A starting cohort of 7 sRA and 8 littermate control mice was used for this study. Eight days after placement of the ICV cannula, the mice were transferred in a home cage into a room dedicated for metabolic measurements where they were given 2 days to acclimate. Their fluid intake and body weight were monitored during the acclimation period. Ten days after ICV cannula placement, the body weight of these mice was measured, they were injected with aCSF ICV in the morning, and then 24 hours later their body weight and water intake were measured. The mice were then injected ICV
with the indicated drug following the order shown in Figure 1B, and then 24-hours later their body weight and water intake were measured. The mice were then given a two day wash out period. This procedure consisting of aCSF injection followed by 24 hour monitoring, ICV drug injection followed by 24 hour monitoring, and two day recovery was repeated as shown in Figure 1B. Concentration of drugs were: BIM (32 μg in 2 μL of DMSO), FR180204 (4 μg in 2 μL of DMSO), Conivaptan (0.8 μg in 2 μL of aCSF), H-89 (4 μg in 2 μL of DMSO), and calphostin c (0.4 μg in 2 μL of DMSO). These mice were not used for any other studies.

Figure 2: ICV BIM and Gö-6976 in sRA and littermate control mice.
Two separate cohorts of sRA (n=4 and n=3) and littermate control mice (n=5 and n=4) were used for the BIM study. Three separate cohorts of sRA and littermate control mice (2-mice per cohort) were used for the Gö-6976 study. 2 μL ICV injections of BIM or Gö-6976 at the doses listed in Figure 2. DMSO was the vehicle. The maximum change in water intake was calculated by curvilinear regression of the change in water intake due to drug from vehicle in each individual mouse. The mean±SEM of the curvilinear regression was calculated and reported. These mice were used for only one study; i.e. a mouse received either an ICV injection of BIM or Gö-6976, but not both. None of the mice were used in any other studies.

Figure 3: ICV AdCRE in ROSA-CAG-LSL-tdTomato mice.
Three separate cohorts of ROSA-CAG-LSL-tdTomato mice (n=3 each) were acutely injected with 1.0 μL of AdCRE ICV. After 4-weeks, they were intra-cardially perfused with 4% paraformaldehyde and the brain was sectioned on a vibratome for imaging.

Figure 4: ICV Ad-DN-PKC-α in sRA and littermate control mice.
Two cohorts of sRA and littermate control mice (n=7 each) were used. Mice were placed into metabolic cages in a room dedicated for metabolic measurements. They were given a two bottle choice between water and 0.15M NaCl. The mice were allowed to acclimate, and then baseline intakes of fluid and food, and body weight were measured. 1 μL of Ad-DN-PKC-α was injected ICV. The mice were placed back into metabolic cages 7 day later, were provided a two-bottle choice of water and saline, were given a day to acclimate, after which their intakes of fluid and food, and their body weight were measured for 2-days. One sRA mouse was eliminated from the study because the fluid intake was found to be an outlier (Grubb’s test, P<0.05).

Figure 5: ICV infusion of BIM in DOCA-salt and control mice.
Two-cohorts of C57BL/6 mice were used (n=22 for DOCA; n=10 for the sham procedure). 10 DOCA-salt and 5 sham mice were infused with aCSF ICV and 12 DOCA-salt and 5 sham mice were infused with BIM ICV. Osmotic mini-pumps were subcutaneously implanted 2 weeks after implantation of the DOCA pellet or sham operation, and an ICV cannula kit was used to infuse drug into the lateral ventricle. Mice were placed into metabolic cages with a two-bottle comparator choice of water and 0.15M NaCl after the 3rd week after DOCA implantation or sham operation. Fluid intakes were measured for 3 days with the first day counted as an acclimation period.
**Figure 6: SFO Cultures**: Cultures of the SFO were obtained from rat newborn pups as previously described with minor modifications.4,5 Briefly, cells from the SFO were incubated in Dulbecco’s modified eagle medium (Life Technologies, Inc.) with 10% bovine serum at 37°C for 9-10 days. PD123319 (an AT2 receptor, 7 μM; Sigma), was applied to the media during treatment. Cultured cells were pre-treated for 30 minutes with vehicle (DMSO in aCSF). Afterwards, the cells were treated for 15 minutes with either DMSO or 10 μM PMA. The cells were fixed and incubated with primary antibody for phosphorylated-PKC-α (1:200; Abcam, Inc., ab76016). Goat anti-rabbit DyLight 488 (1:200; VectorLabs, DI-1488-1.5) in 5% NGS and PBS was used for secondary labeling. The cultures were mounted with media containing DAPI (VectaShield; VectorLabs, H-1200), and were viewed with a Zeiss LSM710.

**Figure 7: ICV PMA in C57BL/6 mice.**
Two cohorts of C57BL/6 mice (n=5 and n=6) underwent surgery for ICV cannula placement. After recovery, these mice were individually housed in a home cage and transferred to acclimate in a room dedicated for metabolic measurements where body weight and water intake was monitored for 2 days. After acclimation, the 1st cohort was ICV injected with vehicle (aCSF) in the morning, and then cumulative water intake at 1-, 5-, 15-, 120-, and 300-minutes and 24-hours was measured. PMA was injected ICV the next morning, and cumulative water intake was measured at the same time periods.

The 2nd cohort of mice were pre-treated with an ICV injection of vehicle (DMSO for BIM), and then 30 minutes were injected ICV with vehicle (aCSF for PMA). Cumulative water intake at 1-, 5-, 15-, and 30-minutes and 24 hours was measured. Next, these mice were pre-treated with DMSO, and then 30 minutes later PMA was injected and cumulative water intake at the same time points was measured. The next morning, the same mice were pre-treated with BIM ICV. 30-minutes later PMA was injected and cumulative water intake at the same time points was measured. A final group received BIM then aCSF to assess the effects of BIM alone.
Supplemental References


Figure S1: Total sodium (A), preference for 0.15 M NaCl (B), food intake (C), body weight (D), and food intake normalized for body weight (E) were measured in sRA (n=13) and littermate control mice (n=14) at baseline (BL) and 9-10 days after ICV Ad-DN-PKC-α. *P<0.05, genotype x virus interaction.