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Selective inhibition of angiotensin receptor signaling through Erk1/2 pathway by a novel peptide

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Abstract

A seven-amino acid peptide (PEP7) is encoded within a short open reading frame within exon 2 (E2) in the 5′-leader sequence (5′LS) upstream of the rat ANG 1a-receptor ($rAT_{1a}R$) mRNA. A chemically synthesized PEP7 markedly inhibited ANG II-induced Erk1/2 activation in cell culture by 62% compared with a scrambled PEP7 (sPEP7) [pErk1/2/Erk1/2 (AU): ANG II, 1.000 ± 0.0, ANG II+PEP7, 0.3812 ± 0.086, ANG II+sPEP7, 1.069 ± 0.18; n = 3]. Under these same conditions, PEP7 had no effect on ANG II-stimulated inositol-trisphosphate production. PEP7 also had no effect on epidermal growth factor- and phorbol methyl ester-induced Erk1/2 activation, suggesting PEP7 selectively inhibits $AT_{1a}R$-mediated Erk1/2 signaling. PEP7 intracerebroventricularly inhibited ANG II-induced saline intake but had no effect on water intake in male and female rats, indicating PEP7 also selectively inhibits the ANG II-Erk1/2 pathway in vivo since saline drinking is Erk1/2-mediated, while water drinking is not. PEP7 inhibition of ANG II-induced saline ingestion was rapidly reversed by a subsequent intracerebroventricular injection of an oxytocin antagonist, suggesting when PEP7 blocks ANG II-stimulated Erk1/2 activation, animals no longer ingest saline to balance the continued water intake, due to the release of oxytocin and its subsequent inhibitory effects on saline drinking. PEP7 also attenuated ANG II-induced increases in arterial pressure by 35% compared with sPEP7 at the same dose. Thus, we have identified a novel peptide encoded

within the rAT$_{1a}$R E2 that selectively inhibits Erk1/2 activation, resulting in physiological consequences for sodium ingestion and arterial pressure that may have implications for treating sodium-sensitive diseases like hypertension and chronic kidney disease.

Keywords: angiotensin II, angiotensin receptor, Erk1/2, sodium intake, PEP7

THE RAT ANGIOTENSIN TYPE 1 receptor (AT$_{1a}$R) plays a critical role in regulating blood pressure and electrolyte homeostasis. This G protein-coupled receptor is encoded by two distinct mRNA transcripts that are synthesized from the AT$_{1a}$R gene by alternative splicing. One of the two transcripts contains exons 1, 2, and 3 (E1, E2, E3), while the other contains E1 and E3. In the E1,2,3 transcript, the 5′ leader sequence (5′LS) comprises E1, E2, and E52 bp of E3 (Fig. 1*A*); in the E1,3 transcript, the 5′LS includes E1 and E52 bp of E3. In both transcripts, the entire coding region and the 3′ untranslated region (3′UTR) exist within E3. Therefore, the two AT$_{1a}$R mRNA transcripts that differ in the 5′LS length encode the same identical AT$_{1a}$R protein.

Despite the identical amino acid sequence of the AT$_{1a}$R in both transcripts, we previously showed that the E1,2,3 mRNA is translated less efficiently than the E1,3 mRNA (21). Furthermore, the E1,2,3 mRNA is associated with lower AT$_{1a}$R densities with less efficient signal transduction compared with the E1,3 mRNA variant (20). Sequence analysis of E2 revealed an upstream ^{108}AUG in excellent Kozak consensus sequence (G/AXXAUGG) (24). This AUG is the start codon of a short open reading frame that encodes a seven-amino acid peptide (PEP7) from $^{-108}$AUG to $^{-87}$TAA, which is in frame with the AT$_{1a}$R coding region (Fig. 1*A*). PEP7 is especially interesting since its amino acid sequence is markedly conserved in rats and mice and humans (Fig. 1*B*).

The AT$_1$R is one of the most versatile members of the G protein-coupled receptor family. Upon ANG II binding, the AT$_{1a}$R mediates signal transduction through multiple signaling pathways, including the phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP$_3$) (32) and extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) (20). These signaling pathways are both G protein-coupled and G protein-independent (16). To determine the effects of PEP7 on these distinct AT$_{1a}$R signaling pathways, this study investigated the pharmacological effects of PEP7 on AT$_{1a}$R-mediated IP$_3$ production and Erk1/2 activation in human embryonic kidney cells transiently expressing the rat AT$_{1a}$R.

To investigate the effects of PEP7 on distinct AT$_{1a}$R signaling pathways in vivo, we studied the effect of PEP7 on water and saline drinking in adult male and female rats, since this is a model in which the stimulatory actions of ANG II depend upon specific signaling cascades (10–12, 14). Indeed, the ability of ANG II to stimulate water drinking can be selectively blocked by inhibitors of PKC activity, while saline drinking is dependent upon Erk1/2 activation. Central administration of ANG II stimulates saline drinking and paradoxically, also increases the local release of oxytocin, which is a peptide that inhibits saline intake (4, 5). We investigated the effects of PEP7 on these apparently competing actions of ANG II in the control of saline ingestion. We also examined the ability of PEP7 to modulate ANG II-induced increases in arterial pressure since AT$_1$R antagonists are widely used clinically to treat hypertension.

MATERIALS AND METHODS

Animals. Male and random cycling female Sprague-Dawley (SD) rats (7–8 wk, 225–275 g) were purchased from Harlan and housed individually under controlled conditions [12:12-h light-dark schedule (0600–1800) at 23°C]. Animals were habituated to these conditions for at least 5 days prior to surgical modification and/or experimentation. Tap water and standard lab chow were available ad libitum. All in vivo animal protocols were approved by the Saint Louis University Animal Care and Use Committee.

PEP7, scrambled PEP7, ANG II, and the oxytocin antagonist. PEP7 (MAGILSG), scrambled PEP7–1 (sPEP7-1) (GIASGLM), and sPEP7–2, (LAMGSIG) were synthesized by LifeTein LLC (Hillsborough, NJ) based on the rat sequence (Fig. 1*B*). ANG II and the oxytocin antagonist, [D-(CH2)5,Tyr(me)2,Orn8]-vasotocin (OVT) were purchased from Phoenix Pharmaceuticals (Burlingame, CA).

Plasmid construction and site-directed mutagenesis. The rat $AT_{1a}R$ transcript containing exons 1, 2, and 3 (5′LS[1,2,3]-CR-3′UTR) was cloned into the pCR3.1 expression vector (Invitrogen).

Cell culture and transfections. Human embryonic kidney (HEK)-293 cells were cultured and transfected with plasmid constructs, as described previously (1).

Erk1/2 activation. Forty eight hours after transfection, HEK-293 cells were preincubated with vehicle, PEP7, sPEP7–1, sPEP7–2, Ro-318220, or AG1478 before treatment with ANG II, epidermal growth factor (EGF), or phorbol methyl ester (PMA). Erk1/2 and pErk1/2 were determined by Western blot analysis, as described previously (37).

IP_3 assay. Twenty four hours after transfection, HEK-293 cells were cultured with 2 μCi/ml myo-[^{3}H]-inositol for 18 h before pretreatment with vehicle or 2.5 μM PEP7 or sPEP7–1 for 3 h, followed by stimulation with ANG II (500 ng·kg^{-1}·min^{-1}) for 5 min. [^{3}H]-IP_3 accumulation was measured as described previously (21).

AT_1R radioligand binding. ^{125}I-[Sar^1,Ile^8]-ANG II competition dose-response curves were conducted with saralasin, PEP7, and sPEP7–1 in isolated rat liver membranes (35), as described previously (18).

Saline and water intake experiments. In the first experiment, vehicle (0.9% NaCl:DMSO, 30:1), or sPEP7–1 (males only) or PEP7 (1.0 nmol icv) were injected (2 μl) via an indwelling cannula in male and female rats, as described previously (39). Ten minutes later, all animals received ANG II (25 pmol/2 μl saline icv) by injection. Thereafter, tap water and 1.5% NaCl drinking bottles were returned to the cages. Water and saline intakes were monitored visually (water bottle gradations = 1 ml). Food was returned to the cages after the 1-h data point was collected.

In the second experiment, male rats were pretreated with PEP7 (1.0 nmol/2 μl icv) and 10 min later, ANG II (25 pmol/2 μl saline icv) was administered as described above, and drinking bottles were returned to the cages. Water and saline intakes were monitored as described above. After

the 30-min data collection time point, vehicle (sterile saline) or the oxytocin antagonist (OVT; 8.7 nmol/2 μl) were administered, and cumulative water and saline drinking were monitored as described previously (5, 39). Food was returned at the 60-min data collection time point.

Measurement of mean arterial pressure. Male SD rats were anesthetized with Inactin, and mean arterial pressure (MAP) was measured through the carotid artery, as described previously (22). Vehicle [dimethylformamide (DMF) or PEP7 and sPEP7–1 (500 ng·kg^{-1}·min^{-1})] were administrated through the jugular vein prior to ANG II treatment. After pretreatment with vehicle (DMF) or 500 ng·kg^{-1}·min^{-1} of PEP7 or sPEP7–1 for 3 h, ANG II (500 ng·kg^{-1}·min^{-1}) was continuously infused for 60 min.

Statistics. The data are expressed as means ± SE. Statistical significance ($P < 0.05$) of the differences between groups was determined by one- or two-way ANOVA with Scheffé's multiple-comparison tests and independent Student's *t*-test where appropriate.

RESULTS

PEP7 inhibits ANG II signaling through the Erk1/2 signal transduction pathway. To determine the effects of exogenous PEP7 on $AT_{1a}R$ signaling, ANG II-induced Erk1/2 activation was investigated in HEK-293 cells transfected with the $AT_{1a}R$ cloned into the pCR3.1 expression plasmid (5′LS[1,2,3]-CR-3′UTR-pCR3.1). ANG II (100 nM) rapidly activated Erk1/2 phosphorylation; the maximum effect on phospho-Erk1/2 (pErk1/2) expression occurred at 5 min, and thus, this time point was used for the PEP7 dose response. Preincubation of the cells with PEP7 markedly attenuated ANG II-induced Erk1/2 activation under conditions in which a sPEP7–1 had no effect (Fig. 2, *A* and *B*). At the highest concentration tested, PEP7 inhibition was maximal after a 3-h preincubation period.

PEP7 does not inhibit ANG II signaling through the PLC signal transduction pathway. To determine whether PEP7 also inhibits ANG II-induced IP_3 formation, HEK-293 cells were transfected with 5′LS[1,2,3]-CR-3′UTR-pCR3.1 and then treated with 100 nM ANG II for 5 min before measuring [^{3}H]-IP_3 production. When the cells were preincubated with PEP7 at the same dose (2.5 μM) and time (3 h) shown to maximally inhibit Erk1/2 activation, we found no effect of PEP7 on [^{3}H]-IP_3 accumulation (Fig. 2*C*).

PEP7 inhibition of Erk1/2 signaling is specific to the $AT_{1a}R$. To determine whether PEP7 inhibition of Erk1/2 signaling is specific to the $AT_{1a}R$, HEK-293 cells were treated with 100 ng/ml of EGF for 5 min or 100 ng/ml of the PKC activator, PMA, for 10 min. Prior incubation of the cells for 15 min with 1 μM AG1478 (an EGF receptor kinase inhibitor) or 10 μM Ro 31–8220 (a PKC inhibitor) inhibited EGF- and PMA-induced Erk1/2 activation, respectively (Fig. 3). In contrast, preincubation with 2.5 μM PEP7, sPEP7–1, or sPEP7–2 for 3 h had no effect on EGF- and PMA-stimulated Erk1/2 phosphorylation.

PEP7 does not inhibit $AT_{1a}R$ binding. Liver membranes were prepared from male SD rats, and radioligand binding studies with ^{125}I[Sar^1,Ile^8]ANG II were conducted in the presence of increasing concentrations of the $AT_{1a}R$ peptide antagonist, saralasin, or PEP7 or sPEP7–1. PEP7 and sPEP7–1

had no effect on ^{125}I[Sar^1,Ile^8]ANG II binding to liver membranes under conditions in which saralasin acted as a potent inhibitor ($IC_{50} = 1.4 \times 10^{-9}$) (Fig. 4).

PEP7 attenuates ANG II-induced saline but not water intake. PEP7 pretreatment (1 nmol icv) 10 min prior had no effect on water intake induced by ANG II administration (25 pmol icv) in both male (Fig. 5*A*) and female (Fig. 6*A*) rats. In contrast, under these conditions, PEP7 pretreatment markedly inhibited saline intake. In male rats, the PEP7 inhibitory effect was observed throughout the 120-min observation period (Fig. 5*B*). Cumulative 24-h water and saline consumption did not differ significantly between groups [Intake (ml): Water (Veh), 21.5 ± 2.9 vs. Water (PEP7), 17.0 ± 2.6; $P = 0.10$; Saline (Veh), 6.2 ± 1.7 vs. Saline (PEP7), 2.9 ± 0.9; $P = 0.10$], although the decrease in saline intake (53%) was much larger than the decrease in water intake (21%). sPEP7 did not alter ANG II-induced water or saline drinking in male rats (Fig. 5, *A* and *B*).

PEP7 also inhibited saline intake in random cycling females; significant differences between vehicle and PEP7-pretreated females were observed at all time points except at 120 min ($P = 0.054$) (Fig. 6*B*), although no significant differences were observed in cumulative saline intake between groups at 24 h ($P = 0.55$) (data not shown).

Oxytocin antagonist administration restores ANG II-stimulated saline intake in PEP7-pretreated rats. The oxytocin antagonist OVT (8.7 nmol icv) failed to alter water drinking in rats pretreated with PEP7 (Fig. 7*A*). However, OVT administration did reverse the PEP7 inhibitory effect on ANG II-induced saline intake (Fig. 7*B*). The reversal was rapid, and the effect was already significant at 10 min following antagonist administration. Cumulative water consumed after 24 h did not differ between groups [intake (ml): Water (PEP7+Veh), 25.1 ± 3.0 vs. Water (PEP7+OVT), 25.7 ± 3.3, $P = 0.91$], and no significant differences were observed in cumulative saline consumed over 24 h in response to ANG II administration between these two groups [Intake (ml): Saline (PEP7+Veh), 3.0 ± 1.1 vs. Saline (PEP7+OVT), 5.3 ± 1.2, $P = 0.18$]. Administration of 8.7 nmol OVT alone either 10 min prior to or 30 min following ANG II injection failed to significantly alter water or saline ingestion in a separate group of animals (data not shown).

PEP7 inhibits acute effects of ANG II on MAP. Acute administration of ANG II (500 ng·kg^{-1}·min^{-1}) via the jugular vein increased MAP in male SD rats by 60.9 ± 1.4 mmHg. Pretreatment with PEP7 at the same dose as ANG II 3 h before ANG II administration inhibited the ANG II-mediated increases in arterial pressure by 35% compared with sPEP7–1 at the same dose (Fig. 8, *A*–*C*). The non-ANG II-treated time control showed that MAP was indistinguishable 5 min prior and 5 min after the period during which ANG II was infused in the treatment groups (data not shown). Furthermore, there was no difference in MAP between the nonpretreated ANG II-stimulated control and the sPEP7-pretreated ANG II-stimulated group either before or after ANG II stimulation (data not shown).

DISCUSSION

The AT_1R mediates signal transduction through multiple signaling pathways (19). In fact, the AT_1R is one of the most versatile members of the seven-transmembrane spanning receptor superfamily in its ability to couple to a myriad of signaling cascades. Much research has been done on the clas-

sic AT_1R IP_3-calcium and PLC-diacylglycerol (DAG)-PKC signaling pathway. Less well understood is how the receptor mediates MAPK signal transduction and transactivation of the EGF receptor. One of the major findings of this paper is that PEP7 inhibited ANG II-induced phosphorylation of Erk1/2 in HEK-293 cells expressing the $AT_{1a}R$ (Fig. 2, *A* and *B*), under conditions in which it had no effect on ANG II-induced IP_3 production (Fig. 2*C*). Thus, PEP7 will be a valuable tool, as the first reported selective inhibitor of the $AT_{1a}R$-Erk signal transduction pathway, to investigate the functional consequences of this nonclassical AT_1R signaling cascade. Furthermore, use of PEP7 will complement studies using Sar^1,Ile^4,Ile^8-ANG II (SII-ANG II), a biased agonist of the $AT_{1a}R$ G protein-independent Erk1/2 pathway, which stimulates AT_1R phosphorylation and coupling to β-arrestin, resulting in a bias toward G protein-independent signaling (6, 17, 37).

The $AT_{1a}R$ can activate Erk1/2 through G protein-dependent mechanisms (Fig. 9). In the classic AT_1R signaling cascade, within seconds, the AT_1R activates PLC producing IP_3 and DAG. DAG can then subsequently activate Erk1/2 via PKC activation (27). The finding that PEP7 did not inhibit Erk1/2 activation via the PKC activator PMA (Fig. 3, *A* and *C*) under conditions in which PMA-induced PKC activation was completely inhibited by the PKC inhibitor Ro-318220 suggests that PEP7 does not inhibit Erk1/2 activation via the PKC pathway. ANG II-induced G protein-mediated signaling pathways leading to Erk1/2 activation also include the Gq/11-PKC-MAPK 1 (MEK) pathway and transactivation of EGF via the EGF receptor and the MEK pathway (19). PEP7 had no effect on AG1478-sensitive EGF-induced Erk1/2 activation (Fig. 3, *A* and *B*), suggesting PEP7 inhibition of Erk1/2 activation is not through transactivation of EGF via the EGF receptor and MEK pathway. ANG II can also induce Erk1/2 activation through G protein-independent signal transduction pathways via β-arrestins and tyrosine kinases (16). Thus, PEP7 may block AT_1R-mediated Erk1/2 activation by interfering with β-arrestin coupling of the AT_1R or by inhibiting further downstream in the β-arrestin pathway.

The finding that PEP7 had no effect on ^{125}I-[Sar^1,Ile^8]ANG II binding to the $AT_{1a}R$ in rat liver membranes (Fig. 4) indicates that the peptide does not inhibit Erk1/2 activation by inhibiting ligand binding to the $AT_{1a}R$; however, it is still possible that PEP7 could act as an allosteric modulator of the receptor to cause a conformational change, resulting in selective inhibition of receptor coupling to the Erk1/2 pathway without altering agonist binding or AT_1R-$G_{q/11}$-PLC coupling to IP_3 and intracellular calcium signaling. The discovery of biased agonists of the AT_1R supports this concept since these peptides induce disparate ligand-induced receptor states of activation (16). For example, the potency of ANG II analogs is markedly increased when position 1 is substituted with the synthetic amino acid L-sarcosine (Sar) (17, 37). Furthermore, distinct differences have been uncovered in the AT_1R structural requirements for G protein-dependent and -independent signaling cascades (2). The carboxy tail of the AT_{1a} R plays a key role in β-arrestin binding and thus in G protein-independent signaling (29), whereas an aspartate (D74) (13) in the second transmembrane plays a crucial role in IP_3 production through an interaction with a tyrosine (Y292) (38) in the seventh transmembrane.

A second major new finding from our study is that we have discovered a pharmacological agent that inhibits ANG II-induced sodium but not water intake (Figs. 5 and 6), which mirrors our in vitro studies and suggests PEP7 is the first pharmacological agent that can selectively inhibit a specific pathway of ANG II signaling in vivo. Emerging studies suggest divergent signaling cascades re-

sult in distinct ANG II-mediated behaviors. Daniels and colleagues have established the importance of Erk1/2 activation in the stimulatory action of ANG II on saline drinking by blocking that transduction cascade with the MAPK inhibitor U0126, but not the PKC inhibitor chelerythrine (10–12). Furthermore, they showed that the biased agonist, SII-ANG II, mimics ANG II stimulation of salt appetite and inhibits ANG II-mediated stimulation of IP_3 with only a minimal enhancement of water intake (11), thereby demonstrating distinctions between these two AT_1R agonists in their receptor interactions. They also demonstrated that ANG II administration increased pERK1/2 levels in the rostral hypothalamus, including the anteroventral paraventricular area, which is an area known to be important not only for water, but also for solute ingestion (7, 34).

We observed that ANG II stimulated saline drinking to a greater extent in females than in males (Figs. 5*B* and 6*B*), but we also observed that during the habituation period prior to testing, ad libitum saline drinking in females exceeded that observed in males. Regardless, PEP7 significantly blocked ANG II-induced saline ingestion in both sexes. It is possible that the ANG II-stimulated saline ingestion and PEP7 inhibitory effects are both sexually dimorphic. Cycling females express lower densities of AT_1Rs than males in key ANG II target tissues (33). Thus, one might expect the magnitude of the effect would be greater in the sex that exhibits lower AT_1R tissue densities. Future studies will address this possible sex difference in greater detail.

ANG II stimulates saline ingestion, but at the same time, appears to increase the central release of oxytocin, a peptide that inhibits salt appetite (5). Blackburn et al. (3, 4) have provided convincing evidence for the physiological relevance of those actions of oxytocin, but if this is the case, how could these apparently conflicting actions be balanced in vivo? We hypothesized that the direct action of ANG II (on yet to be identified neuronal populations in the forebrain) in stimulating saline drinking predominates over oxytoxin-mediated inhibition of salt appetite. In this scenario, the peptide balances the ingestion of solute-free water with appropriate amounts of osmotically active ingestate. When the Erk1/2 signal transduction cascade activated by ANG II via the β-arrestin pathway is blocked, as here by PEP7 administration, the inhibitory action via oxytocin release would then hold saline drinking in check, even in the face of continued water ingestion and potential hemodilution. If this is the case, then antagonism of the action of the endogenous oxytocin released in response to ANG II should reverse the inhibition, and the animals should then consume saline. This is what we observed, and, in fact, the reversal was robust and rapid and appeared within the first 5 min following OVT administration (Fig. 7). Importantly, these studies provide insight into the balance of stimulatory and inhibitory effects of downstream signaling circuits activated by ANG II. Thus, we are hypothesizing that ANG II acting via cells in which the MAPK pathway is initiated drives a predominating stimulatory effect on saline ingestion and that this effect overwhelms the peptide's action to release oxytocin. In the absence of MAPK activation (i.e., PEP7 inhibition), the effect of oxytocin to hold saline intake in check is uncovered. Our results support the hypothesis that ANG II stimulation of oxytocin release is via the G protein-dependent signaling pathway and, therefore, not affected by MAPK inhibition.

Our third major finding in this study is that PEP7 can lower the arterial pressure response to ANG II stimulation (Fig. 8). This observation suggests ANG II-dependent Erk1/2 activation contributes to blood pressure regulation. Hypertension and associated diseases, including stroke (9), atherosclerosis (36), and chronic kidney disease (8), remain the number-one cause of death throughout

the world (25, 28). In the United States, more than 50% of hypertensive patients do not have their blood pressure well controlled. Suboptimal blood pressure control is, in part, due to poor patient compliance because of the adverse side effects (e.g., dizziness, headache, cough, and sexual dysfunction) associated with the widely prescribed angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs). Lack of control is also due to the frequent need for more than one medication to achieve target blood pressure (23), and it is especially common in patients with salt-sensitive hypertension. Thus, the discovery of PEP7 may lead to novel therapeutics for attenuating hypertension.

The finding that the synthesized peptide has selective inhibitory effects on AT_1R-mediated Erk1/2 activation both in vitro and in vivo under conditions in which scrambled peptides have no effect strongly suggests the short open reading frame (sORF)-encoding PEP7 plays a physiological role in modulating AT_1R actions. Furthermore, the fact that the encoded peptide is highly conserved across species (Fig. 1*B*) lends supports that, indeed, there is a physiological role for the PEP7 sORF; however, even if the peptide is not translated in vivo, the fact that PEP7 has pharmacological actions in vivo on sodium appetite and blood pressure makes this peptide an interesting newly discovered modulator of the critically important renin-angiotensin system. Given the hydrophobic nature of the peptide, we hypothesize that PEP7 administered exogenously crosses the cell membrane and interferes with AT_1R coupling to the non-G protein-dependent β-arrestin-2 pathway either directly or indirectly.

Elucidating the actions of a novel peptide encoded within the 5′LS of the $AT_{1a}R$ has ramifications for other seven-transmembrane-spanning receptors since sORFs are widely encountered in this protein superfamily (26, 31), as well as in other key proteins like protooncogenes, signaling molecules, and immune mediators (40). Yet, the actions of peptides encoded within these sORFs remain poorly understood. Forty percent of all prescription drugs are targeted toward the seven-transmembrane-spanning receptor class of proteins (15), which is more than any other family of proteins (30). Therefore, this research may have implications for developing novel therapeutics across a large family of drug targets.

Perspectives and Significance

Our finding that PEP7 uniquely inhibits ANG II-induced Erk1/2, but not IP_3 signaling, in vitro and in vivo raises the possibility that PEP7 mimetics could have therapeutic potential. A selective inhibitor of this AT_1R signaling cascade would offer a therapeutic profile that is distinct from ACEi or ARBs. It could be used as a monotherapy or in combination with ARBs to produce synergistic effects and reduce off-target adverse events by lowering therapeutic dosages. Moreover, a selective inhibitor of sodium intake would provide novel approaches to the regulation of salt-sensitive disease states such as salt-sensitive hypertension and renovascular hypertension. In addition to the potential clinical impact, a selective inhibitor of the AT_1R-Erk1/2 pathway would be a highly valuable and novel pharmacological tool for elucidating mechanisms of AT_1R actions since PEP7 is the first identified selective inhibitor of this pathway. Lastly, it is of note that upstream sORFs are present in most G protein-coupled receptors, oncogenes, growth factors, and kinases. Thus, understanding the biology of PEP7 could open the door to discovering a novel mechanism for regulating these critical signaling proteins.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.L., G.L.C.Y., R.C.S., W.K.S., and K.S. conception and design of research; J.L., G.L.C.Y., H.J., D.Z., W.Z., and W.K.S. performed experiments; J.L., G.L.C.Y., H.J., W.Z., and W.K.S. analyzed data; J.L., G.L.C.Y., and H.J. prepared figures; G.L.C.Y., R.C.S., W.K.S., and K.S. interpreted results of experiments; H.J., R.C.S., and K.S. edited and revised manuscript; W.K.S. drafted manuscript; W.K.S. and K.S. approved final version of manuscript.

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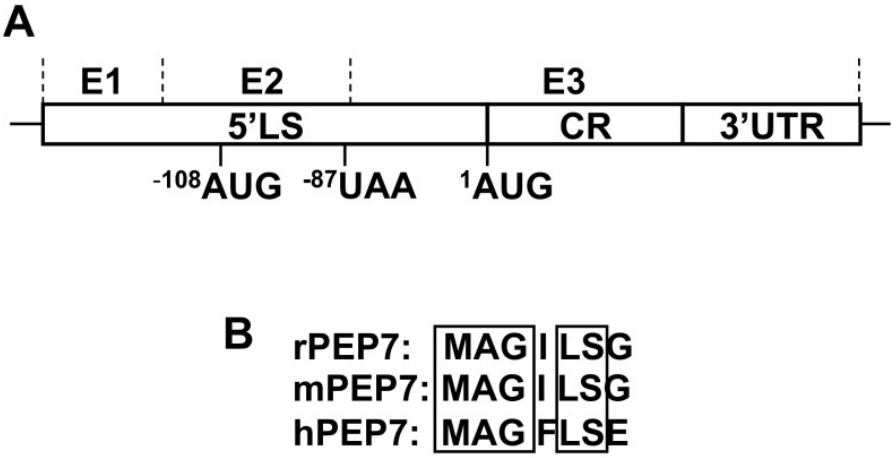
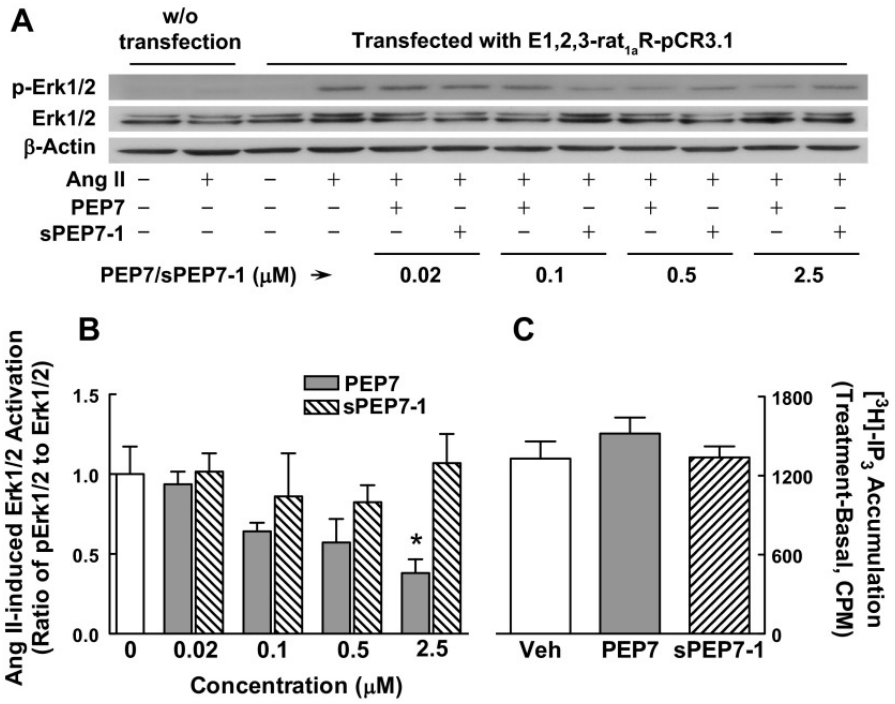
Figures and Tables

Fig. 1.



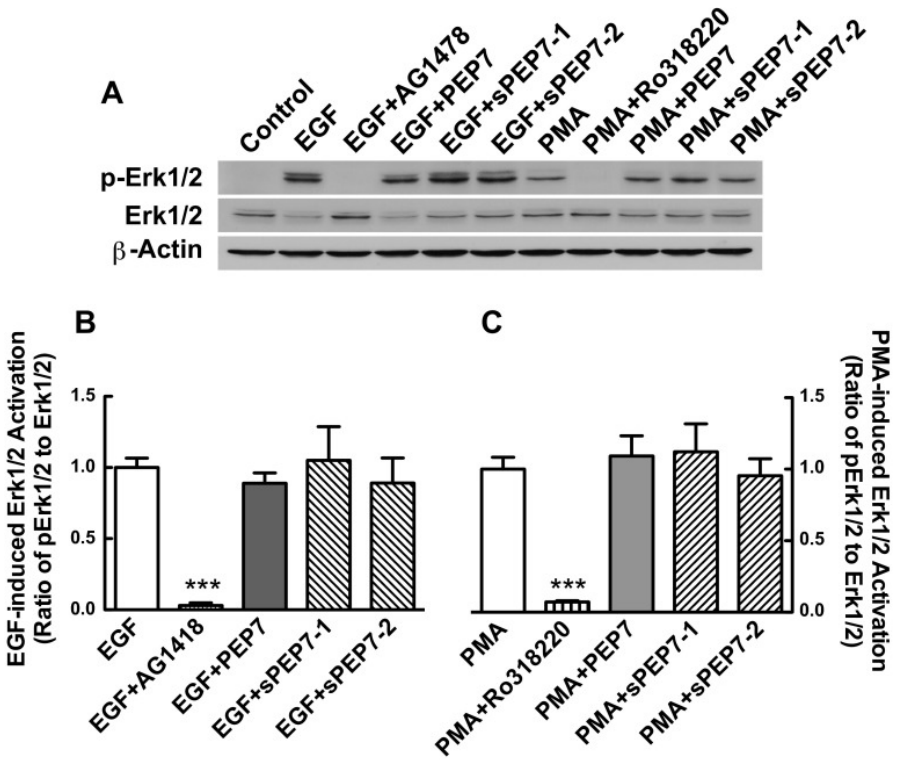
Schematic of the rat $AT_{1a}R$ mRNA and PEP7 sequence homology. *A*: the exon AT1aR gene encodes three exons (E). E2 includes a short open reading frame that codes for PEP7 in the 5′ leader sequence (5′LS) upstream of the receptor coding region (CR) and 3′-unstranslated region (3′UTR). *B*: sequence homology of rat (r), mouse (m), and human (h) PEP7.

Fig. 2.



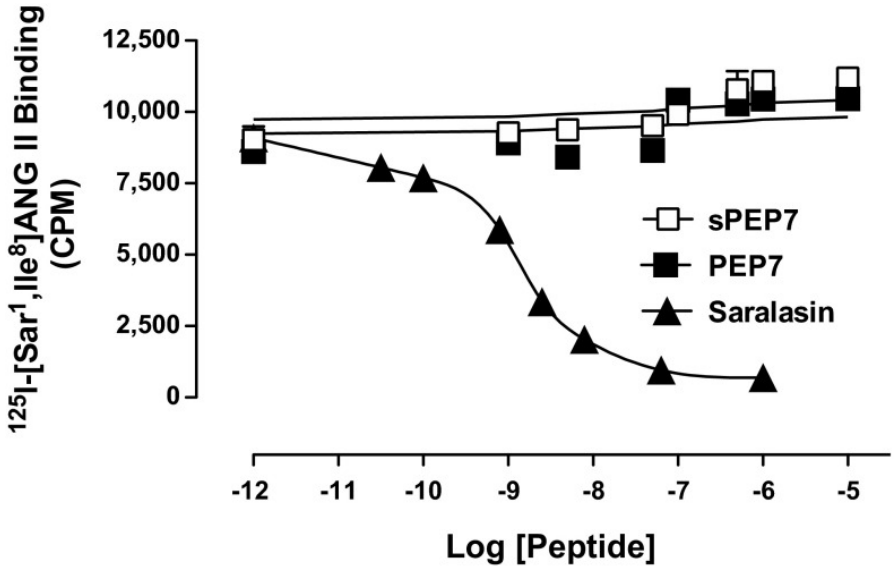
Effect of PEP7 on $AT_{1a}R$-mediated Erk1/2 activation. *A* and *B*: HEK-293 cells were transfected with E1,2,3-$AT_{1a}R$-pCR3.1. Transfected cells were preincubated for 3 h with increasing concentrations of PEP7 or sPEP7–1 before incubating cells with ANG II (100 nM) for 5 min and then immediately assessing Erk1/2 activation via Western blot analysis. *A*: Western blot of Erk1/2, pErk1/2, and β-actin. The gel is representative of three experiments. *B*: quantitation of the Western blot data. **P* < 0.05 vs. sPEP7–1. *C*: transfected HEK-293 cells were pretreated with PEP7 or sPEP7–1 at 2.5 μM for 3 h before treatment with ANG II (100 nM) for 5 min and then immediately measuring [^{3}H]-IP_3 accumulation. The data are expressed as the means ± SE; *n* = 3.

Fig. 3.



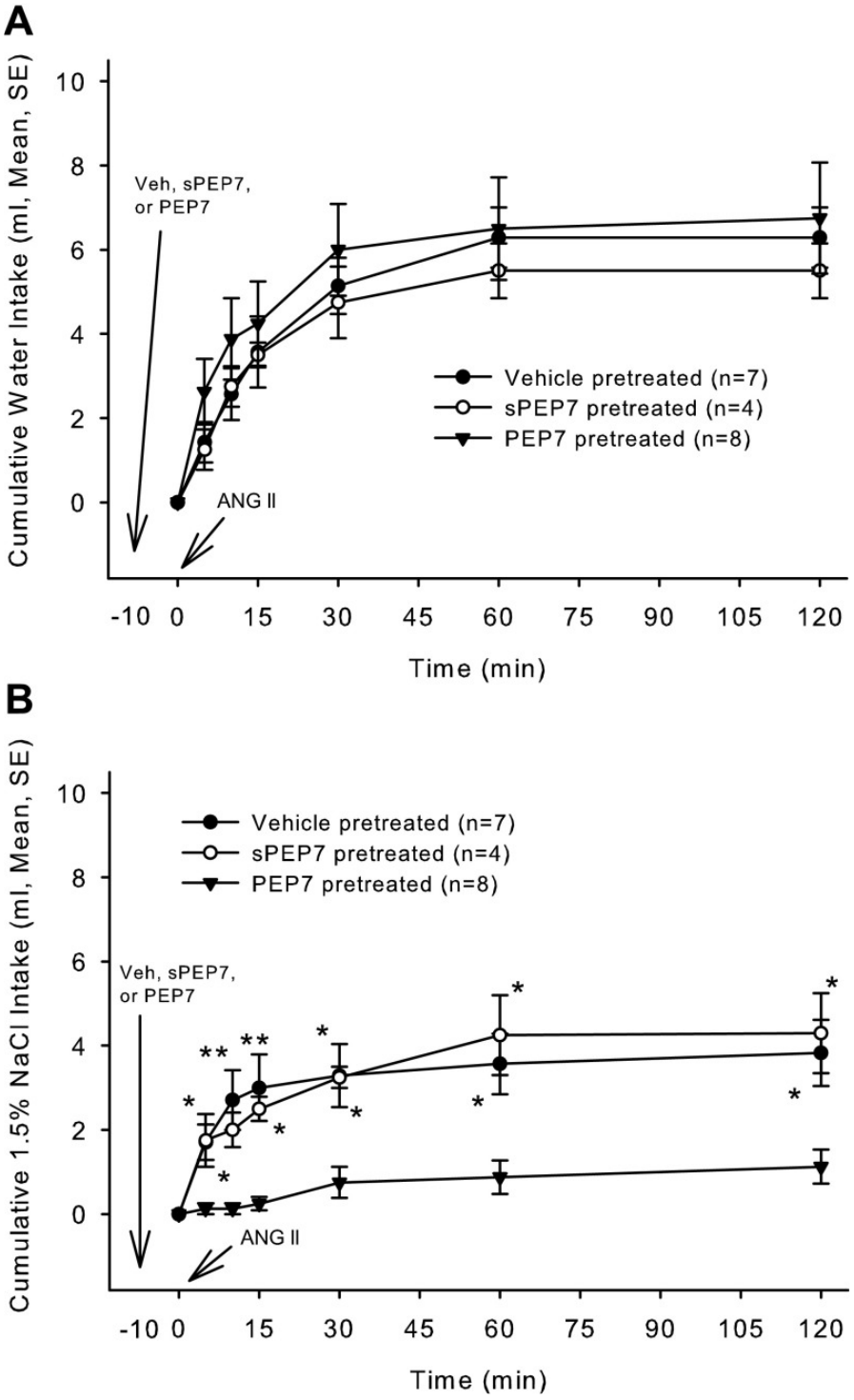
Effect of PEP7 on EGF- and PMA-induced Erk1/2 activation. HEK-293 cells were preincubated with PEP7 or two sPEP7 variants (sPEP7–1 and sPEP7–2) at 2.5 μM for 3 h or with the EGF inhibitor AG1478 (1 μM) or PKC inhibitor Ro318220 (10 μM) for 15 min and then treated with EGF (100 ng/ml) or PMA (100 ng/ml) for 5 or 10 min, respectively; $n = 3$. *A*: Western blot of Erk1/2, pErk1/2, and β-actin. The gel is representative of three experiments. *B*: quantitation of the EGF Western blot data. ***$P < 0.001$ vs. EGF by one-way ANOVA. *C*: quantitation of the PMA Western blot data. ***$P < 0.001$ vs. PMA.

Fig. 4.



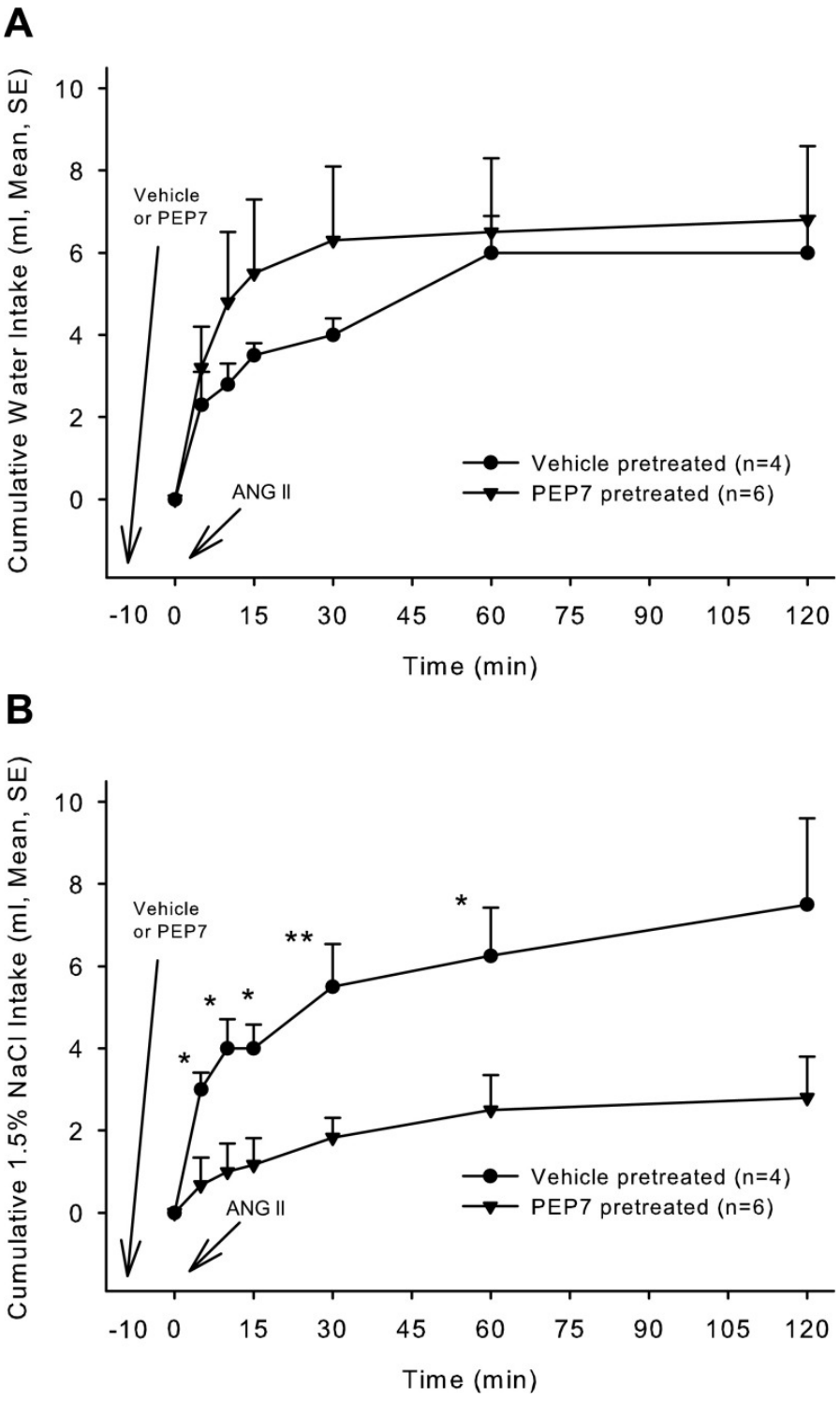
Effect of PEP7 on ^{125}I-[Sar^1,Ile^8]ANG II binding to the $AT_{1a}R$. Rat liver membranes were incubated with ^{125}I-[Sar^1,Ile^8]ANG II and increasing concentrations of saralasin (▲), PEP7 (■), or sPEP7–1 (□). The data are representative of two experiments performed in triplicate.

Fig. 5.



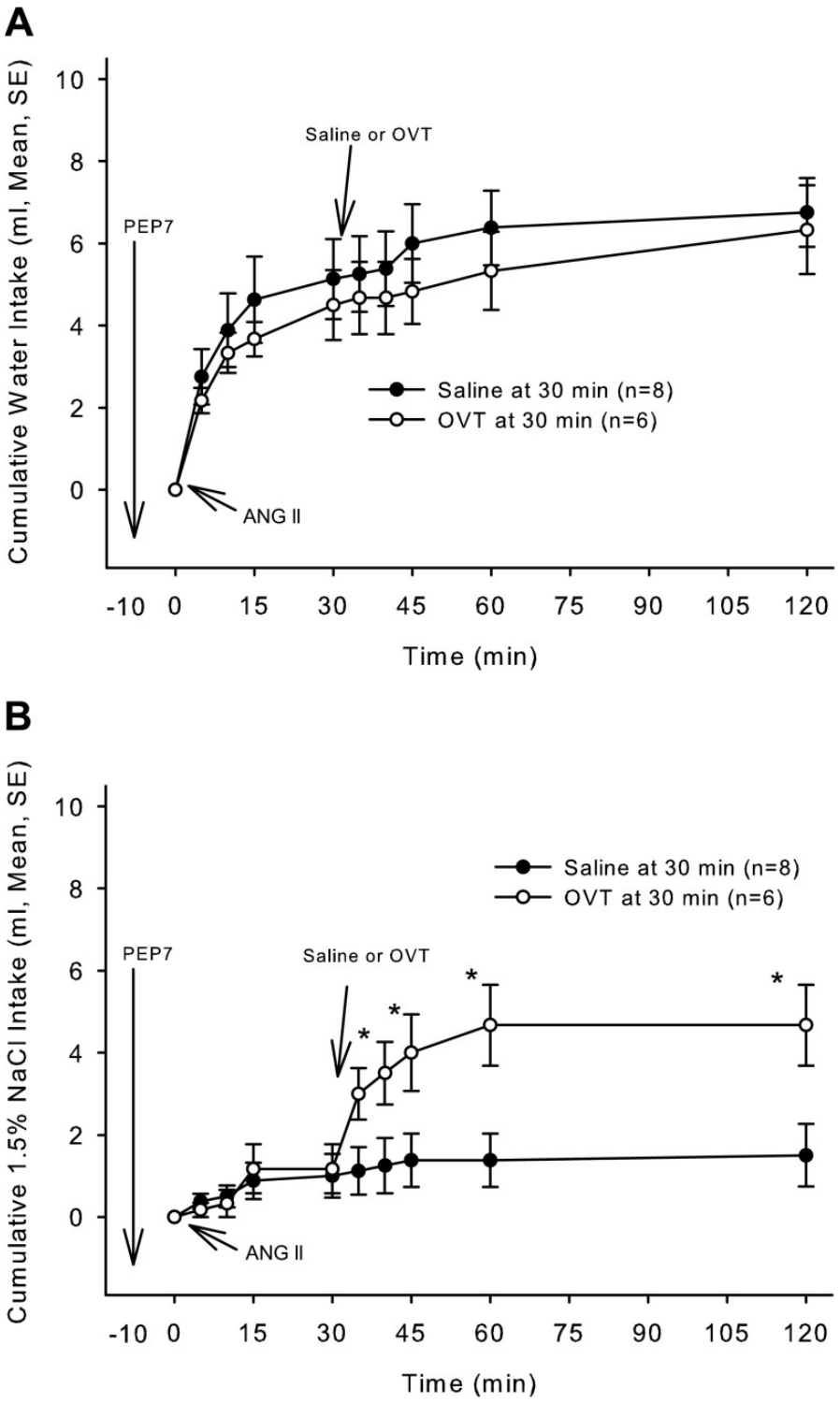
Effect of PEP7 on in vivo ANG II action in male rats. Water (*A*) or saline (*B*) intake in response to ANG II after pretreatment with PEP7 (▲), sPEP7–1 (○), or vehicle (Veh; ●). $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. PEP7 pretreated animals. (ANOVA, Scheffé's multiple comparisons).

Fig. 6.



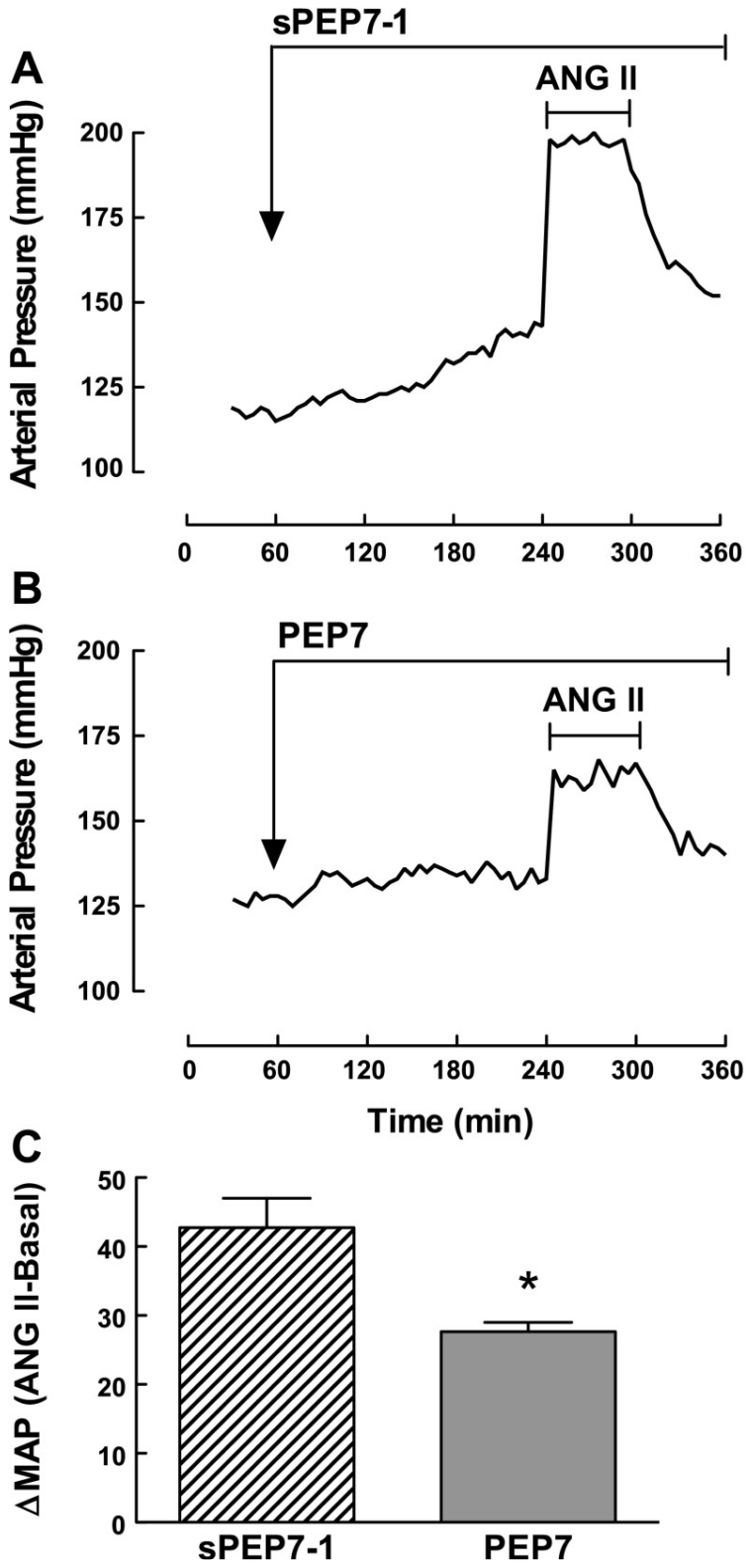
Effect of PEP7 on in vivo ANG II action in female rats. Water (*A*) or saline (*B*) intake in response to ANG II after pretreatment with PEP7 (▲), or vehicle (closed circle). **P* < 0.05 or ***P* < 0.01 vs. PEP7-pretreated animals (independent Student's *t*-test).

Fig. 7.



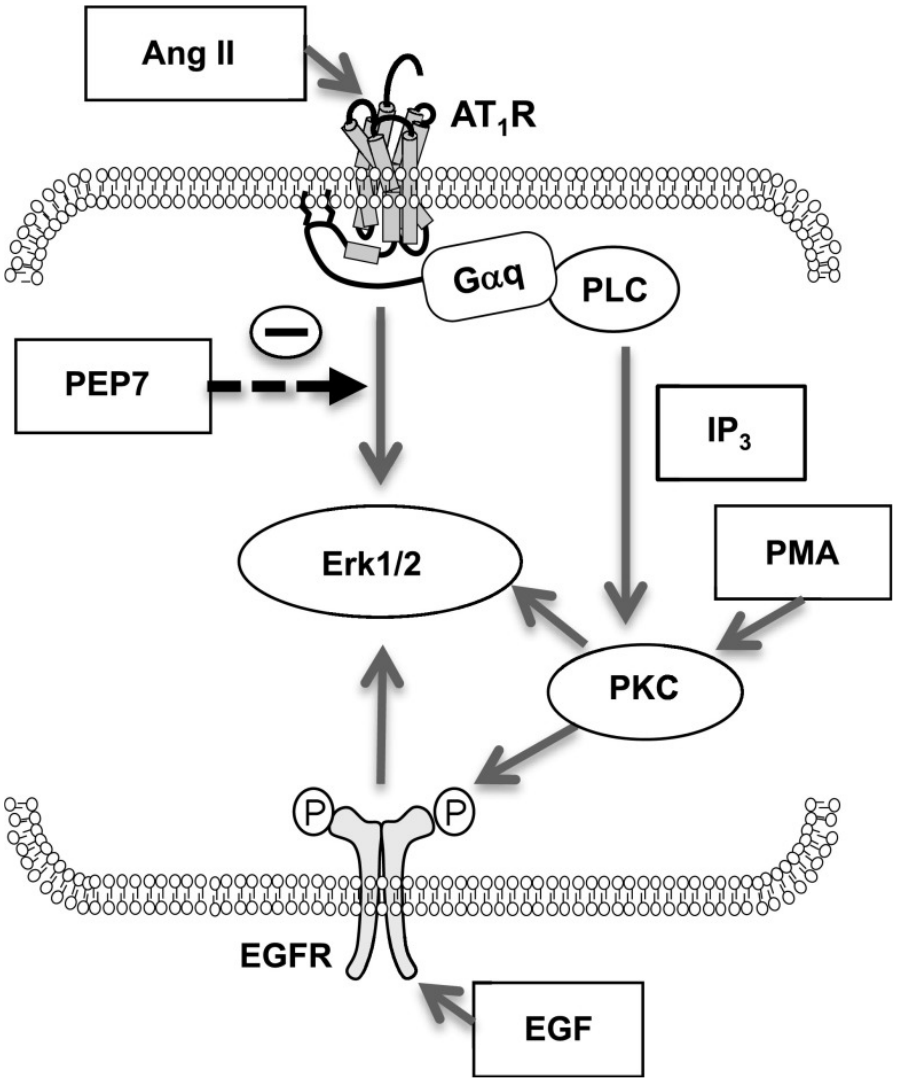
Effect of [D-(CH2)5,Tyr(me)2,Orn8]-vasotocin (OVT), an oxytocin antagonist, on PEP7 inhibition of ANG II-induced saline and water intake. Water (*A*) or saline (*B*) intake was measured in male rats in response to ANG II after pretreatment with PEP7 followed by saline vehicle (●) or OVT (○) treatment; **P* < 0.05 vs. saline (PEP7+OVT) (independent Student's *t*-test).

Fig. 8.



Effect of PEP7 on ANG II-induced increases in arterial pressure. Anesthetized male rats were pretreated with sPEP7–1 (n = 3) (A) or PEP7 (n = 4) (B) at 500 ng·kg^{-1}·min^{-1} for 3 h before ANG II was infused for 30 min at 500 ng·kg^{-1}·min^{-1}. The arterial pressure data are representative of 3 or 4 experiments each. C: quantitation of the peak MAP. The data are expressed as the means ± SE; *P < 0.05 vs. sPEP7–1.

Fig. 9.



Proposed site of PEP7 inhibition of the AT_1R-Erk1/2 signaling cascade. PEP7 inhibits Erk1/2 activation through the G protein- and PKC-independent β-arrestin pathway.